1-2012

Dynamic Endothelialization of Aortic Heart Valve Scaffolds

Richard Pascal
Clemson University, Rpascal@clemson.edu

Dynamic Endothelialization of Aortic Heart Valve Scaffolds

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

---

by
Richard Pascal III
August 2012

Accepted by:
Dr. Dan Simionescu, Committee Chair
Dr. Agneta Simionescu
Dr. Karen Burg

ABSTRACT

Cardiovascular disease is the number one killer worldwide affecting both the heart and blood vessels. Valvular heart disease can arise from calcification, and structural deterioration resulting in a stenotic or regurgitant valve incapable of proper function. With approximately 275,000 valve replacements performed annually worldwide, the need for replacement heart valves is well established. Currently, treatment of valvular heart disease is limited to two options (mechanical and bioprosthetic). Both replacement valves have their own drawbacks, which have driven research in the bioengineering field to focus on the development of a tissue engineered heart valve (TEHV) capable of growth and self-repair.

A major hurdle in the creation of a viable TEHV lies in the need for a confluent surface layer of endothelial cells (EC) prior to implantation. ECs are needed in TEHVs because they provide a natural non-thrombogenic surface, and a permeability barrier between blood and the vessel wall. One major step in the TEHV paradigm lies in the development of a means for delivering cells to a heart valve scaffold with the purpose of achieving this confluent cell layer. As it stands now there is no recognized standard for EC seeding, though researchers have developed a number of different devices and protocols attempting to successfully achieve uniform cellular attachment.

The goal of this Master's thesis research was to design and create a dynamic cell-seeding device capable of seeding cells onto the surface of a decellularized porcine aortic heart valve scaffold. Once developed, the dynamic seeding device was to be used to create a protocol for optimizing cellular attachment and confluence on the heart valve

surface. Additionally, following cell seeding, the next step in the TEHV paradigm is mechanical preconditioning prior to functional implantation. Utilizing a pulsatile heart valve bioreactor, seeded scaffolds were subjected to mechanical forces for the purpose of studying cellular retention and the effects of mechanical stimuli on cell morphology. Analysis of cellular attachment, retention, and viability was done through the use of Live/Dead Assay and Scanning Electron Microscopy (SEM). The results of both Live/Dead and SEM showed that the dynamic seeding device is capable of seeding porcine aortic endothelial cells onto the surface of aortic heart valve scaffolds and that the cells could be retained on the surface after undergoing physiologic bioreactor conditioning. The cells were found to respond to the conditioning, changing morphology and aligning in response to these mechanical forces.

ACKNOWLEDGMENTS

I owe an immeasurable amount of gratitude to both Drs. Dan and Aggie Simionescu for taking me in and sharing their expertise and knowledge with me. I can't go without thanking them for their patience over the years. Their vision and leadership has and will continue to impact my life. Together you have both created something very special in the BTRL and I am extremely thankful to have been afforded the opportunity to be a part of it.

I'd also like to thank Dr. Jeremy Mercuri for teaching me how to do research and for being the role model scientist he's been for the entire BTRL since day 1. Thanks J for all the mentoring and friendship over the past several years. I'd like to acknowledge and give a big thanks Lee Sierad for his friendship and guidance both in- and outside the lab. Without your selfless willingness to help me at all hours and your spiritual influence this would not have been possible.

I want to acknowledge my fellow BTRL members for all of their help and friendship. The support of each of you is greatly appreciated. Thanks to my other committee member Dr. Karen Burg, and the BioE department chair Dr. Martine Laberge.

I'd like to thank my parents and my brother for their patience, love, and support over the years. In the toughest of times, their words of encouragement were solely responsible for carrying me through.

Finally, I would like to acknowledge the funding sources for my research NIH grant R21EB009835 (to Dr. Agneta Simionescu) and NIH grant 5R01HL093399 (to Dr. Dan Simionescu).

TABLE OF CONTENTS

Page
ABSTRACT i
ACKNOWLEDGMENTS iii
TABLE OF CONTENTS iv
LIST OF TABLES vii
LIST OF FIGURES viii
INTRODUCTION AND BACKGROUND 1
1.1 Cardiac Anatomy and Physiology 1
1.2 Heart Valve Anatomy and Physiology 4
1.3 Heart Valve Cell Types and Their Function 7
1.4 Heart Valve Pathology 9
1.5 Current Valve Replacement Treatments 10
1.6 Current Research and Future Direction 14
1.7 Cell Seeding Techniques 19
1.7.1 Static Seeding 19
1.7.2 Dynamic Seeding 21
1.7.3 Perfusion Seeding 25
PROJECT APPROACH AND RATIONALE 28
MATERIALS AND METHODS 30
3.1 Scaffold Preparation 30
3.1.1 Fresh Tissue Harvesting 30
3.1.2 Heart Valve Decellularization 30
3.1.3 Fixation 31
3.2 Porcine Aortic Endothelial Cell Culture 34
3.2.1 Fibronectin (FN) Cell Culture Flask Coating Protocol 34
3.2.2 Cell Culture and Confluent Cell Passaging 34
3.2.3 Cell Counting 35
3.3 Dynamic Seeding Device Design 35

3.4 Porcine Aortic Endothelial Cell Seeding Studies with Dynamic Seeding Device 49
3.4.1 Study 1: Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study) 49
3.4.2 Porcine Aortic Endothelial Cell Seeding for Studies 2-6 53
3.4.3 Study 2: Initial Low Concentration Cell-Seeding Study 53
3.4.4 Live/Dead Assay 55
3.4.5 Study 3: Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity 56
3.4.6 Study 4: Computer Control For Increased Coverage and Variable Seeding-Time Study 59
3.4.7 Scanning Electron Microscopy (SEM) Sample Preparation 64
3.4.8 Study 5: Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions 66
3.4.9 Study 6: Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions 70
EXPERIMENTAL RESULTS AND DISCUSSION 73
4.1 Study 1: Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study) 73
4.2 Study 2: Initial Low Concentration Cell-Seeding Study 75
4.3 Study 3: Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity 77
4.4 Study 4: Computer Control For Increased Coverage and Variable Seeding-Time Study 79
4.5 Study 5: Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions 83
4.6 Study 6: Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions 100
CONCLUSIONS 116
5.1 Study 1: Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study) 116
5.2 Study 2: Initial Low Concentration Cell-Seeding Study 116
5.3 Study 3: Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity 117

5.4 Study 4: Computer Control For Increased Coverage and Variable Seeding-Time Study 117
5.5 Study 5: Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions 117
5.6 Study 6: Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions 118
5.7 Final Conclusions and Recommendations for Future Studies 118
APPENDICES 121
6.1 Appendix A 122
6.1.1 Porcine Heart Valve Decellularization Protocol 122
6.1.2 Porcine Heart Valve Tissue Fixation Protocol 123
6.2 Appendix B 124
6.2.1 Fibronectin Coating of Cell Culture Flask Protocol 124
6.2.2 Cell Culture and Confluent Cell Passaging Protocol 124
6.2.3 Cell Counting Protocol 126
6.2.4 Porcine Aortic Endothelial Cell Seeding Protocol 126
6.3 Appendix C 129
6.4 Appendix D 131
6.4.1 Live/Dead Assay Protocol 131
6.4.2 Scanning Electron Microscopy (SEM) Sample Preparation Protocol 131
REFERENCES 133

LIST OF TABLES

Table Page

Table 1: Pressure Values Over 1-week Conditioning 83
Table 2: Pressure Values Over 2-week Bioreactor Retention Study 101

LIST OF FIGURES

Figure Page

Figure 1: Blood Flow Through the Heart (Scheve 2008) 3
Figure 2: Fibrosa Collagen Bundles and Ventricularis Surfaces 6
Figure 3:Trilaminar Aortic Valve Structure and Matrix Composition and Function Relationship (Mendelson and Schoen 2006) 6
Figure 4: Resident Cells in a Heart Valve Cusp. Movat's Pentachrome stain. Elastin (Dark Red), Collagen (Yellow), Cells (Dark Blue), and Proteoglycans (Light Blue) (Sierad et. al 2010). 8
Figure 5: Heart Valve Prosthesis (Jaron et al. 2008) 12
Figure 6: Statically Seeding Cells onto a Scaffold (Villalona et al. 2010) 20
Figure 7: Dynamic Seeding of Cells on a Scaffold (Villalona et al. 2010) 22
Figure 8: Acrylic Ball Dynamic Rotational Device (Lueders et al. 2006) 24
Figure 9: Free Floating Mount Rotational Seeding Device (Gulbins et al. 2005) 25
Figure 10: Perfusion Cell Seeding Example (Villalona et al. 2010) 26
Figure 11: Magnetic Cell Seeding (Shimizu et al. 2007) 26
Figure 12: Porcine Aortic Heart Valve Root. Steps Through Scaffold Preparation Protocol. .Top Row= View From the Aorta. Bottom Row=View From Ventricle 32
Figure 13: View of Aortic Heart Valve After Opening by Dissection. Steps Through Scaffold Preparation Protocol. 33
Figure 14: Porcine Aortic Endothelial Cell Culture. From Left to Right, Sterilized Cell Seeding Chamber, Cell Culture Medium, Trypsin, T-150 Culture Flasks with ECs 35
Figure 15: Heart Valve Bioreactor Mounting Ring and Modifications for Dynamic Seeding Device 37
Figure 16: Heart Valve Bioreactor Mounting Ring and Modifications for Dynamic Seeding Device 38
Figure 17: Dynamic Seeding Device Chamber 40
Figure 18: Dynamic Seeding Device Heart Valve Chamber Lid 41
Figure 19 Acrylic Dynamic Cell Seeding Chamber 44
Figure 20: Designed Flow of Cells Through the Heart Valve Scaffold. Blue=Seeding Chamber, Green=Lid, Orange & Purple= Mounting Rings, Red= HV Scaffold, Black= Endothelial Cells 45
Figure 21: Aluminum Seeding Chamber Mounting A-Frame 46
Figure 22: Aluminum Shaker Frame and Stepper Motor 47
Figure 23: Overview of Device in Incubator with Computer Control 48
Figure 24: Study 1: Defining an Axis of Rotation. Looking at Flow Around the Valve.. 50
Figure 25: Study 1: Hand Rotation of Seeding Chamber 51
Figure 26: Study 1: Black Pepper Visually in Suspension during Rotation 52
Figure 27: Mounting of Heart Valve Scaffold onto Mounting Ring 54
Figure 28: Study 2 Rotational Device Setup with Drill Motor and Power Supply 55
Figure 29: Sterile Mounting of Decellularized Heart Valve Scaffold into Cell-Seeding Mounting Rings 57

Figure 30: Mounting Heart Valve into Cell-Seeding Mounting Rings..... 58
Figure 31: Study 3: Mounted Heart Valve in Dynamic Seeding Chamber 59
Figure 32: Upgraded Dynamic Seeding Device Overview..... 60
Figure 33: Dynamic Seeding Device Software Interface 61
Figure 34: Study 4 Variable Seeding Time Elapse..... 63
Figure 35: Anatech USA Hummer 6.2 Sputter Coating (Anatech USA 2012) 64
Figure 36: Hitachi TM-3000 Scanning Electron Microscope (Hitachi High-technologies 2010) 65
Figure 37: Heart Valve Bioreactor System. A) Medium Flow Through System B) Schematic Overview of Entire Bioreactor System: 1) Three-Chambered Heart Valve Bioreactor, 2) Pressurized Compliance Chamber, 3) Medium Reservoir Pump, 4) Sterile Filter for Gas Exchange, 5) One-way Valve, 6) Pressure Retaining Valve, 7) Webcam, and Ventilator Pump (Air Pump). C) Heart Valve Bioreactors with Mounted Cell-Seeded Heart Valves in the Cell Culture Incubator (Sierad et al. 2010)..... 67
Figure 38: Cell-Seeded Heart Valve Mounted in the Heart Valve Bioreactor. Top Image: Closed Valve Under Diastolic Pressure. Bottom Image: Open Valve Under Systolic Pressure..... 68
Figure 39: Macroscopic View of Mounted Heart Valves Under Rotation 71
Figure 40: Study 1 Black Pepper Sequential Rotation Images..... 74
Figure 41: Study2: Live/Dead Assay; Axial Cusp=Fibrosa and Ventricularis 1. Non-Axial Cusps=Fibrosa and Ventricularis 2 & 3. Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red.....**Error! Bookmark not defined.**
Figure 42: Study 3: Live/Dead Assay; Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red. A. Non-Coronary Cusp B. Coronary Cusp (Radial) C. Coronary Cusp (Axial) D. Coronary Artery (Axial) E. Coronary Sinus (Axial) F. Aortic Lumen G.Aortia, Cusp Junction. 78
Figure 43: Study 4 Live/Dead Assay; Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red 80
Figure 44: Study 4: 4Hr sample SEM..... 81
Figure 45: Study 4 6hr Sample SEM 82
Figure 46: Study 5 6 Hr Dynamic Seeding and 1 Hr Static incubation Live/Dead (Coronary Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red 84
Figure 47: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation Live/Dead (Coronary Ventricularis). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 85
Figure 48: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation Live/Dead (Coronary 2 Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 86
Figure 49: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation Live/Dead (Non-Coronary Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red 87

Figure 50: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation Live/Dead (Non-Coronary Ventricularis). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 88
Figure 51: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation Live/Dead (Aortic Sinus). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 89
Figure 52: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation SEM (Fibrosa).... 90
Figure 53: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation SEM (Fibrosa).... 91
Figure 54: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation SEM (Fibrosa).... 92
Figure 55: Study 5 6Hr Dynamic Seeding and 1 Hr Static incubation SEM (Fibrosa).... 93
Figure 56: Study 5 Post 1 Week Mechanical Conditioning Live/Dead (Non-Muscle Coronary). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red.... 94
Figure 57: Study 5 Post 1 Week Mechanical Conditioning Live/Dead (Muscle Coronary). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 95
Figure 58: Study 5 Post 1 Week Mechanical Conditioning Live/Dead (Non-Coronary). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 96
Figure 59: Study 5 Post 1 Week Mechanical Conditioning SEM (Fibrosa)..... 97
Figure 60: Study 5 Post 1 Week Mechanical Conditioning SEM (Fibrosa)..... 98
Figure 61: Study 5 Post 1 Week Mechanical Conditioning SEM (Fibrosa)..... 99
Figure 62: Study 6 Post 6 Hr Dynamic Seeding and Overnight Static Incubation Live/Dead + DAPI. Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red. DAPI Stain Binds DNA and Cell Nuclei Fluoresce Blue 102
Figure 63: Study 6 Post 6 Hr Dynamic Seeding and Overnight Static Incubation SEM (Fibrosa)..... 103
Figure 64: Study 6 Post 6 Hr Dynamic Seeding and Overnight Static Incubation SEM (Fibrosa)..... 104
Figure 65: Study 6 Post 2 Week Mechanical Conditioning Live/Dead (Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red..... 105
Figure 66: Study 6 Post 2 Week Mechanical Conditioning Live/Dead (Ventricularis & Aortic Lumen). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red 106
Figure 67: Study 6 Post 2 Week Mechanical Conditioning SEM (Fibrosa)..... 109
Figure 68: Study 6 Post 2 week Mechanical Conditioning SEM (Fibrosa)..... 110
Figure 69: Study 6 Post 2 Week Mechanical Conditioning SEM (Fibrosa)..... 111
Figure 70: Study 6 Post 2 Week Mechanical Conditioning SEM (Fibrosa)..... 112
Figure 71: Study 6 Post 2 Week Mechanical Conditioning SEM (Fibrosa)..... 113
Figure 72: Study 6 Post 2 Week Mechanical Conditioning SEM (Fibrosa)..... 114
Figure 73: Study 6 Post 2 Week Mechanical Conditioning SEM (Fibrosa)..... 115

CHAPTER ONE

INTRODUCTION AND BACKGROUND

1.1 Cardiac Anatomy and Physiology

The heart, in conjunction with the vascular system, performs the vital role of sustaining life. The heart supplies oxygenated blood and nutrients concomitant with the removal of both deoxygenated blood and waste from the tissues of the body. The heart is structurally composed, in large part, of myocardium, or specialized cardiac muscle tissue. The myocardium provides the contractile forces necessary for the pumping of blood throughout the entire cardiovascular system. In performing the vital role of providing nutrients and oxygen to the body, it is imperative that the cardiovascular system maintains a unidirectional flow. A series of valves found in strategic anatomic locations within the heart are responsible for the maintenance of this unidirectional flow. As pressure gradients across the valves shift with contraction, they function to either open to permit forward flow of blood through the vasculature or close to prevent backflow of blood. Without proper functioning heart valves, a pathology commonly known as valvular heart disease, physiological failure of the cardiovascular system occurs.

Taking a closer look at the anatomic structure of the heart (Figure 1), the organ is divided into four chambers; the left atrium, right atrium, left ventricle, and right ventricle. Furthermore, the heart is a dual circuit pump divided into the pulmonary and systemic circuits. The pulmonary circuit is responsible for the pumping of blood from the heart through the lungs where oxygenation occurs prior to flow back to the heart.

The process begins as deoxygenated blood, high in carbon dioxide, returns to the heart from the body via the vena cava. It enters the right atrium and flows through the tricuspid valve into the right ventricle. Following atrial systole, or contraction of the atrium, the remaining blood is forced from the atrium into the right ventricle. Subsequent contraction of the right ventricle forces closure of the tricuspid valve, and the opening of the pulmonary valve allowing blood to flow through the pulmonary trunk and into the pulmonary arteries. The pulmonary arteries become heavily branched into small diameter thin walled capillaries capable of transluminal gas exchange. Alveolus, or tiny air sacs, in the lungs facilitate the exchange of carbon dioxide for oxygen with red blood cells. As oxygenated red blood cells leave the lungs, the capillaries combine into larger diameter vessels and ultimately into the pulmonary vein before returning to the heart. As the blood returns to the heart, it enters the left atrium before flowing through the mitral valve into the left ventricle. Atrial systole again forces the remaining blood from the atrium into the left ventricle. Filling with blood, ventricular systole occurs and the left ventricle contracts. As pressure in the ventricle increases, the mitral valve is forced closed preventing retrograde flow. This increased ventricular pressure also forces the aortic valve open allowing the blood to leave the heart through the aorta bound for the tissues of the body. As the oxygenated blood flows through the aorta it continues through the arteries branching into the systemic capillaries. Red blood cells pass single file through the capillaries where they exchange oxygen for carbon dioxide with the tissues. The deoxygenated blood flows through the systemic veins merging into the larger diameter vena cava before entering the right atrium of the heart to complete the cycle.

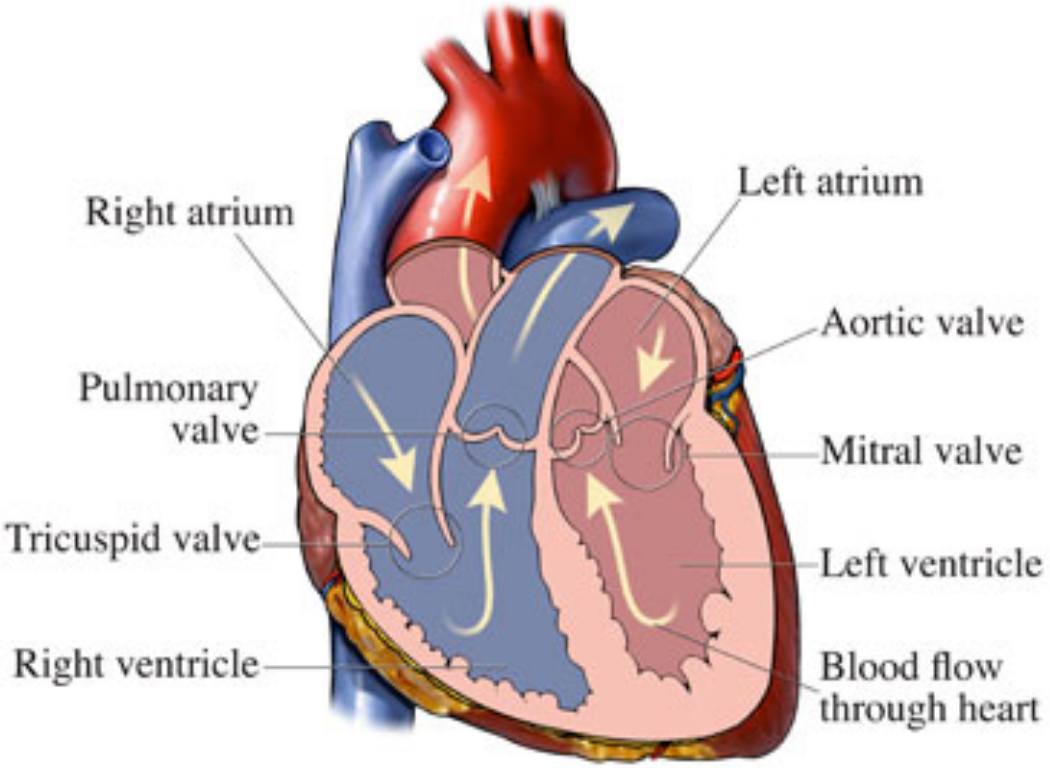


Figure 1: Blood Flow Through the Heart (Scheve 2008)

1.2 Heart Valve Anatomy and Physiology

In addressing valvular heart disease, a thorough understanding of the heart valve anatomy is at the very core of developing a solution. Heart valves are complex in structure and function and anatomically vary between each of the four valves. As with all things in anatomy, form follows function and the heart valves are no exception. The varying structure of each valve is dictated by their individual function, and the mechanical forces which each experience. The atrioventricular valves reside between the atrium and ventricle of both the left and right sides of the heart (Figure 1). They prevent retrograde flow from the ventricle back into the atrium. Both the left and right ventricles function to pump blood from the heart through the systemic and pulmonary circuits and in doing so operate at much greater pressures than their atrial counterparts. The variance of this pressure gradient makes the atrioventricular valves susceptible to prolapse into the atrium. Negating this effect is the chordae tendineae, which attach to the cusp and papillary muscles in the ventricles and under ventricular systole become tight preventing their inversion.

The semilunar valves, located between the ventricles and the aorta and pulmonary arteries have vastly differing anatomic structures from their atrioventricular counterparts. The semilunar valves are composed of three cusps arranged in a tri-leaflet structure. Each leaflet attaches to the annulus of the valve conduit. The point where two leaflets come together on the annulus is known as the commissure. During diastole, pressure forces the valve closed and three cusps come together at a point of coaptation where

sufficient overlap of the leaflets results in the prevention of retrograde blood flow (Mendelson and Schoen 2006).

The stresses experienced by the semilunar valves are among the highest found in the human body. Systolic and diastolic pressures experienced by the pulmonary valve are about 40/25 millimeters of mercury respectively. Specifically, the forces exhibited on the aortic heart valve are characterized as the greatest and most complex stresses within the cardiovascular system (Butcher and Nerem 2007). Unlike the rest of the cardiovascular system, which experiences its greatest stresses when the heart is in systole, the aortic valve is under its maximum pressure when the heart is in diastole (Butcher and Nerem 2007). Systolic and diastolic pressures experienced by the aortic valve are 120/80 millimeters of mercury respectively. These stresses include shear stress due to blood flow, flexure during opening and closing, and tension upon closure (Mendelson and Schoen 2006). The heart valve leaflet is composed of a tri-layered structure with two fibrous layers separated by an internal spongy gelatinous layer. The superior fibrous layer is known as the fibrosa while the inferior and thinnest layer of the heart valve is called the ventricularis. Between the two resides the spongiosa layer.

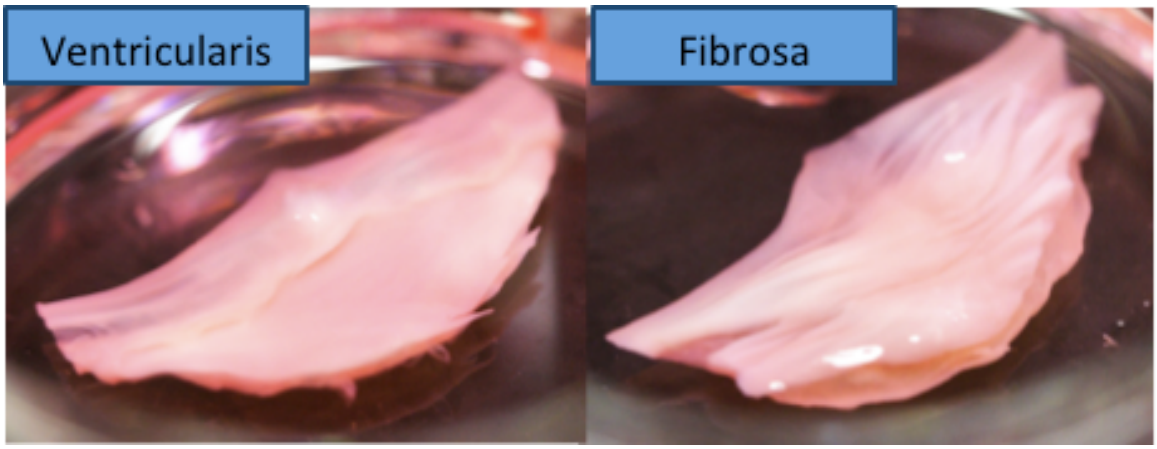


Figure 2: Fibrosa Collagen Bundles and Ventricularis Surfaces

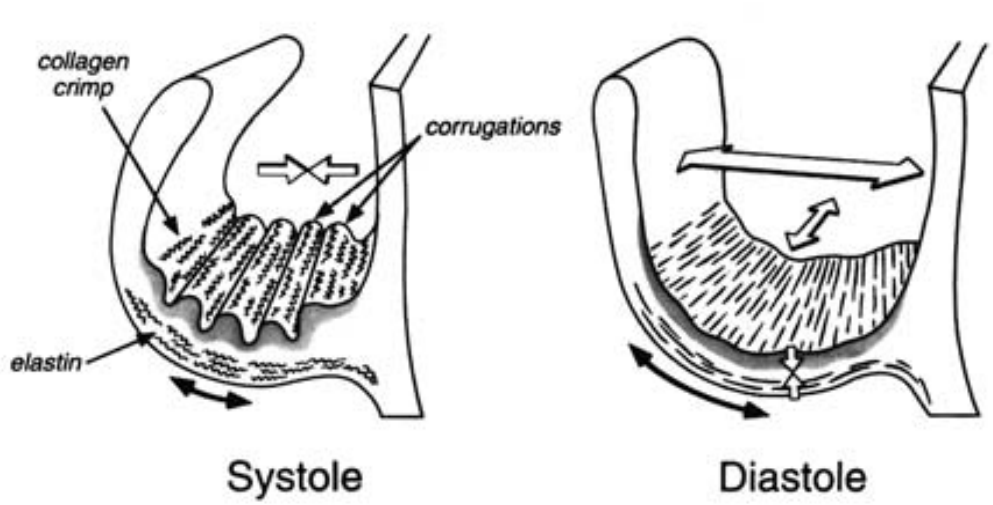


Figure 3:Trilaminar Aortic Valve Structure and Matrix Composition and Function Relationship (Mendelson and Schoen 2006)

The fibrosa layer is comprised mostly of dense, organized collagen with collagenous bundles macroscopically visible and oriented in a circumferential direction (Figure 2). These collagen bundles and their positioning and orientation functions to provide tensile strength to the heart valve leaflet (Mendelson and Schoen 2006). The

spongiosa is mostly composed of proteoglycans and glycosaminoglycans (GAGs) and loosely packed collagen. Proteoglycans and GAGs function to keep the layer hydrated giving the spongiosa its amorphous gelatinous structure (Mendelson and Schoen 2006). The nature of the spongiosa's structure provides for both compressive strength as well as protecting against shear stresses between the fibrosa and ventricularis layers. The ventricularis layer contains a majority of the elastin found in the heart valve and functions to prevent permanent deformation of the leaflets (Apte et al. 2011).

Under native function, the heart valve experiences both compressive and tensile forces which the cusps collagen, elastin, and GAG's function together in distributing these forces across the heart valve (Figure 3). The mere content of elastin, collagen, and GAG's alone does not dictate the proper mechanical function of the heart valve. Equally as important to its function is the organization of these building blocks within the cusps.

1.3 Heart Valve Cell Types and Their Function

Characteristic of all things living and seen throughout all tissues of the body, resident cells constantly react to their environment. Mechanical forces and molecular cues are responsible for directing these actions. There are two major cell types that populate the heart valve. Valvular interstitial cells (VICs), most abundant and myofibroblastic in nature reside within the fibrosa and spongiosa layers (Figure 4) (Butcher and Nerem 2006; Apte et al. 2011; Chester and Taylor 2007). Reacting to environment stimuli VICs synthesize extracellular matrix proteins and mediate the remodeling of the matrix (Butcher and Nerem 2007).

The second cell type found within the heart valve is the valvular endothelial cell (VEC). While VICs reside within the heart valve and are responsible for matrix maintenance, VECs form a confluent layer on the surface of the valve known as the endothelium (Figure 4). VECs perform a number of vital roles similar to vascular endothelial cells found lining blood vessels throughout the body.

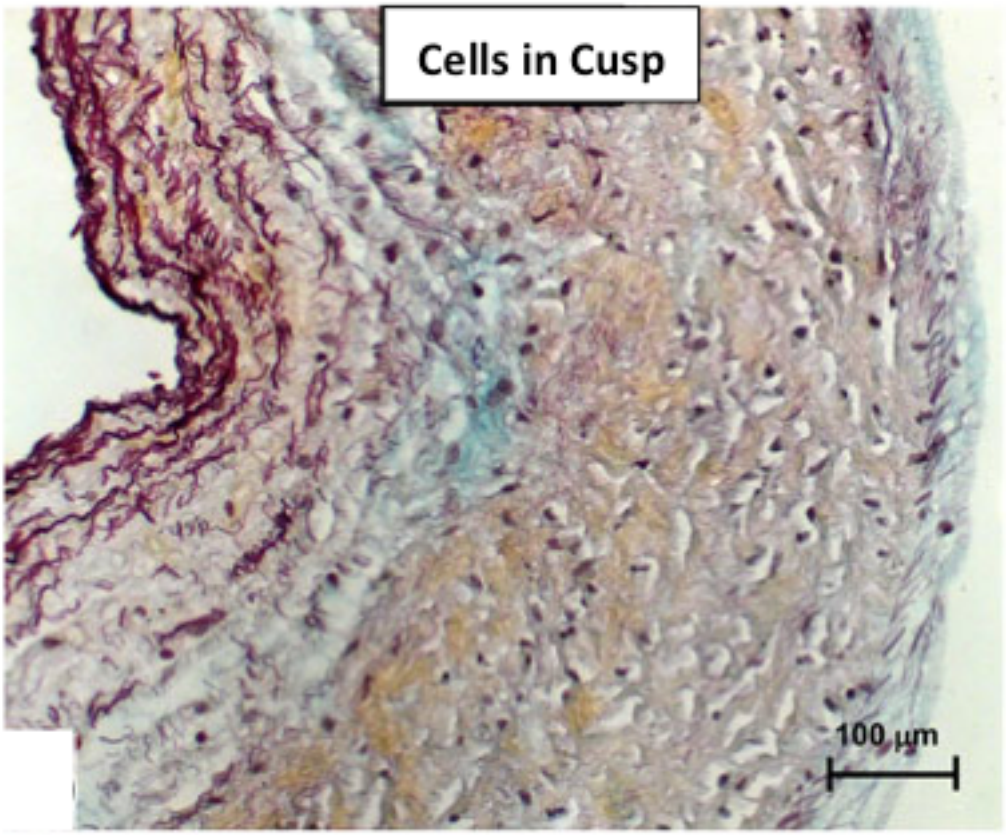


Figure 4: Resident Cells in a Heart Valve Cusp. Movat's Pentachrome stain. Elastin (Dark Red), Collagen (Yellow), Cells (Dark Blue), and Proteoglycans (Light Blue) (Sierad et. al 2010)**.**

Endothelium is composed of specialized endothelial cells whose morphology resembles elongated ellipsoids, showing a strong correlation to their subjected mechanical stimuli. Anatomically speaking, form follows function and due to the differing mechanical environments experienced by VECs and vascular ECs, their anatomic

orientations differ. Vascular ECs experience a primary circumferential strain as well as a unidirectional shear force due to blood flow. In response to these forces, vascular ECs appearing morphologically elongated, align in a direction parallel to the flow of blood through vessel walls and perpendicular to the circumferential strains experienced. VECs, influenced by their highly specific mechanical environment and radial principle strain, also exhibit an elongated morphology. In contrast however, VECs align circumferentially on the valve surface, perpendicular to the direction of blood flow. This has been found to be true for both the fibrosa and ventricularis surfaces of the valve cusps (Butcher and Nerem 2007). VECs maintain the non-thrombogenic blood-tissue interface, and regulate immune and inflammatory reactions. Forming the barrier between blood and underlying tissues, VECs play a major role in the body's response to injury via the coagulation cascade. Wearing away of or damage to the endothelium leads to initiation and up-regulation of clotting factors. Any damage to the endothelium amplifies the shear stresses experienced on the heart valve creating turbulent flow and exposing underlying matrix proteins to blood. This exposure increases the likelihood of platelet adhesion and coagulation up regulation in response (Lichtenberg et al. 2006; Butcher and Nerem 2007; Sierad et al. 2010). Breakdown of the endothelium has been found to in large part be the initiating cause for pathological valve failure (Butcher and Nerem 2007).

1.4 Heart Valve Pathology

Heart valve pathology takes on many forms and can range from defects from birth to degenerative valves. Some specific types of problems include: regurgitation, stenosis, and atresia. Often times these complications occur together. Regurgitation occurs when the

heart valve is unable to close fully, allowing the blood to flow backwards. The most common form of regurgitation is mitral valve prolapse, which occurs when the valve's physical condition is weak and they flop backwards or bulge. Regurgitation leads to disruption in the unidirectional flow of the cardiovascular system causing inefficiency and consequentially making the heart work harder. Stenosis is when the valves have thickened, stiffened, or fused together. As a result, not enough blood can flow through the valve. This condition often coincides with regurgitation as stiffened valves function abnormally under pressure unable to form a good seal. Atresia is when the valves are partially fused closed, which also prevents proper blood flow (Heart Lung and Blood Institute 2011). These pathologic ailments all lead to decreased heart efficiency resulting in greatly increased stress and work for the heart. Ultimately valvular heart disease must be treated through a valve replacement surgery.

1.5 Current Valve Replacement Treatments

Valvular heart diseases affect 15 million people in the United States alone and resulted in 90,000 valve replacement surgeries in 2009 (Tillquist and Maddox 2011). Currently the two types of valves on the market for replacing diseased and defective heart valves are mechanical and bioprosthetic (Figure 5). In treating the defective heart valves through surgical means, a patient and their physician must weigh the pros and cons of both types in deciding which is best.

Mechanical valves have been used as replacements since the 1950's beginning with the ball in cage model that has evolved into today's tilting-disc and bileaflet versions. Current mechanical valves are made of pyrolitic carbon, a man-made material

that exhibits a high degree of durability and resistance to wear resulting in a long lifetime of 20-30 years of proper function. However, due to the nature of their design, mechanical valves tend to elicit a non-laminar flow resulting in higher than normal shear stresses and subsequent increased risk for thrombosis on the valve surface (Apte et al. 2011; Tillquist and Maddox 2011). This thrombotic accumulation on the surface leads to an increased risk of embolism. Due to this increased risk and the severity of its consequences, a patient opting for a mechanical replacement valve must undergo a lifelong regimen of anticoagulation therapy, most often with a vitamin K antagonist such as warfarin (Tillquist and Maddox 2011). Use of anticoagulation therapy doesn't come without its own side effects, including the increased risk of bleeding as the body's natural coagulation process is hindered. This therapy also necessitates significant changes in the patient's lifestyle including abstinence from contact sports, other high-risk physical activities, as well as the use of other medications that may interact with the anticoagulant drugs (Tillquist and Maddox 2011). Pregnancy and surgical procedures may require the suspension of this anticoagulation therapy and must also play a major role when making the decision between mechanical and bioprosthetic valves (Tillquist and Maddox 2011).

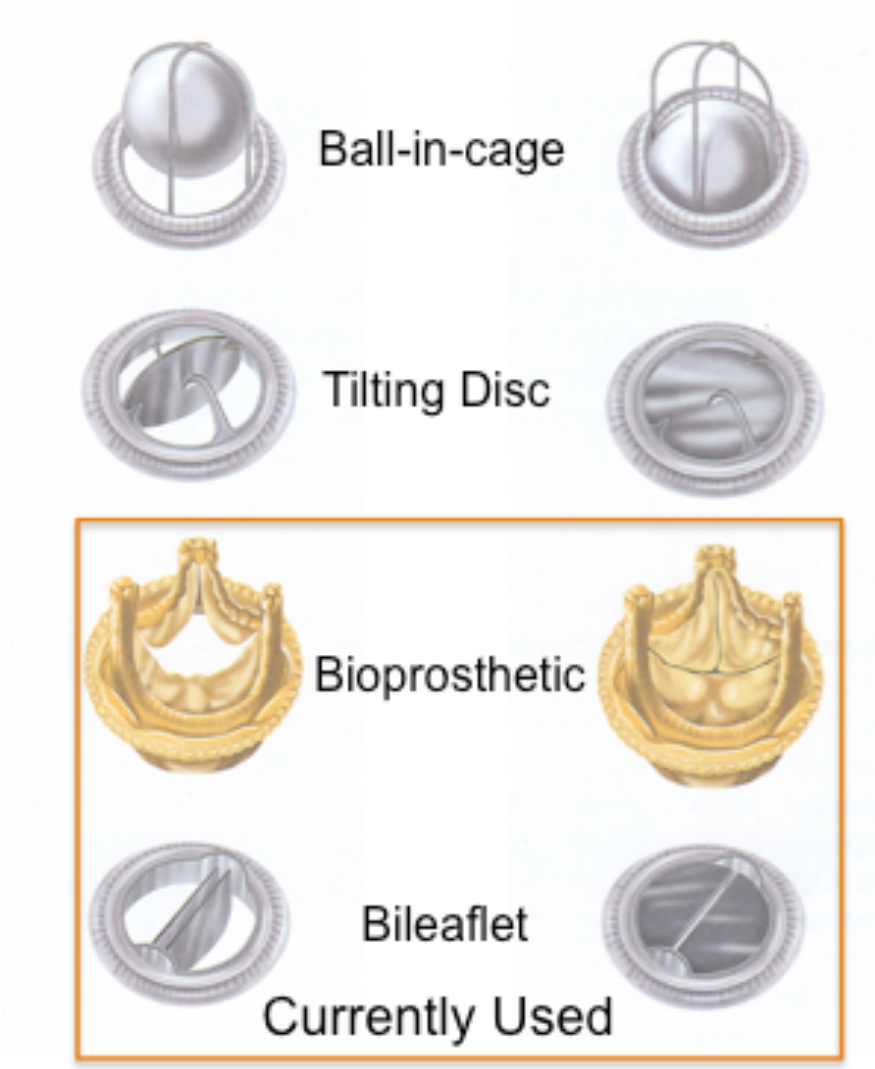


Figure 5: Heart Valve Prosthesis (Jaron et al. 2008)

Bioprosthetic heart valves used in valve replacements composed of biological tissues of either a porcine or bovine origins are known as xenografts. The xenograft tissue is chemically fixed, fashioned into a similar shape of the native valve, and mounted to a suture ring for ease of surgical implantation. One major advantage of bioprosthetic valves compared to their mechanical counterparts is the lack of need for the aforementioned anticoagulation therapy leading to a decreased risk of bleeding

complications post implantation (Tillquist and Maddox 2011). Bioprosthetic valves possess their own unique disadvantages. They are much less durable than mechanical valves and have a reduced implant lifetime of 10-15 years. This reduction in viability is attributed to both calcium and lipid deposition on the implant surface which leads to deterioration both structurally and mechanically (Butcher and Nerem 2007). This mechanical deterioration results in loss of proper function leading to a reoperation and subsequent replacement. As with any surgical procedure, there are always inherent risks involved making this the major downside of bioprosthetic valves.

Ultimately the decision on the type of heart valve a patient will receive resides on a number of factors that must be weighed by the patient and taken into account. The number one factor is most often the age of the patient as the difference in the lifespan of each valve is significant and a reoperation is always undesirable. A second major factor to be considered is the lifestyle of the patient and the effects of an anticoagulant regimen. Recommendations by physicians as to the type of valve a patient should receive follows the general trend that patients 60+ years of age are candidates for a bioprosthetic as the average population lifespan wouldn't warrant the need for a reoperation to replace a deteriorated valve. For patients under 60 years of age, a mechanical heart valve is the general recommendation as average life expectancy of the valve exceeds that of the bioprosthetic valve and greatly reduces the risk of reoperation in younger patients (Tillquist and Maddox 2011). These guidelines must be further analyzed on a case-by-case, patient-to-patient basis. Patients experiencing other co-morbidities must consult with a physician in assessing their risks that they may make an informed decision.

As noted, neither option is perfect and both are less than ideal. Younger patients are at the greatest disadvantage with their choice negated entirely and left to a mechanical heart valve and consequently a long life of anticoagulant treatment (Hecker and Birla 2007). Adolescents and younger patients often lead more active lifestyles and anticoagulant therapy puts serious limits on many lifestyle choices. Neither option is able to adequately accommodate the growth of younger patients hearts leaving implanted valves to hinder blood flow and require several re-operations (Mirensky et al. 2010).

1.6 Current Research and Future Direction

Current research working on the development of the next generation heart valve replacement is centered on the premise of a tissue engineered heart valve. The ideal heart valve prosthesis should be non-inflammatory, non-thrombogenic, capable of growth, repair, and remodeling, durable for the life of the patient, cheap and readily available, and anatomically tailored to the needs of the individual patient (Apte et al. 2011). For the successful replication of function of a replacement tissue engineered valve, researchers must not only model the implant after the native valve on a macroscopic level but replication of the heart valve extracellular matrix (ECM) components is necessary in reproducing the hemodynamics of the native heart valve. In the paradigm of tissue engineered heart valves, replication of this native endothelium and underlying vascular interstitial cells combined with native extracellular matrix composition would prove invaluable in the successful production of a living implant capable of growth, self-repair, and native hemodynamic properties.

Combining scaffolds with cells, through a process known as cell "seeding," is at the core of many tissue engineering strategies. Due to the role of matrix components in native heart valve function, scaffold development lies at the foundation of a successful tissue engineered heart valve. Scaffolds are expected to offer mechanical and chemical signals and induce cells to adhere, proliferate, differentiate and excrete ECM and new tissue substitutes. In selecting scaffold materials, several requirements must be met. An ideal scaffold should be both physically and chemically stabile, easily obtained and sterilized, possess suitable mechanical properties. In addition, materials should be safe, non-toxic, non-pyrogenic, non-allergenic, non-deformable, non-carcinogenic as well as possess tissue, blood, and immunological compatibility. The most ideal scaffolds should contain bioactive sites and offer biological signals promoting cell growth, ECM formation, degradation, and tissue regeneration and repair at the molecular level. A friendly interaction between scaffold and seeded cells benefiting tissue regeneration is the ultimate desire. In studying potential scaffolds, there are several qualities to be evaluated including degradation rates, biocompatibility, mechanical properties, and ease of sterilization (Dong et al. 2008).

A final TEHV product should exhibit the following characteristics: 1) good compatibility with the host's cardiovascular system; 2) adequate mechanical strength and durability; 3) excellent hemodynamic performance without the need for anticoagulation therapy; 4) absence of immunogenic and/or inflammatory reactions; 5) most importantly, the ability of self growth and repair. Current issues holding up progress in successful scaffold development include the inability to endure systematic circulation pressures, lack

of bioactive sites for coordination of cell-scaffold interactions, and inability to maintain endothelial cell adherence thus exposing underlying matrix to inflammatory cell and platelet adhesion resulting in thrombosis and implant degradation (Dong et al. 2008)

Researchers are looking at different potential scaffold materials of which fall into one of two major categories: synthetic bio-absorbable polymers or natural biological materials including decellularized biological matrices.

Synthetic scaffolds are created using bio-absorbable polymers such as polyglycolide (PG), polylactide (PL), poly (D, L-lactide-co-glycolide, copolymer of PG and PL) (PLG), polyhydroxyalkanoates (PHAs) and polyelthylene glycol (PEG) and are most widely used due to ease of production and ability to modify physical and chemical properties (Dong et al. 2008; Mendelson and Schoen 2006). Synthetic scaffolds run into challenges with regulating cell adhesion as well as 3-dimension tissue re-organization (Mendelson and Schoen 2006). Major drawbacks with synthetic scaffolds lies in their lack of bioactive signals and biocompatibility making them less desirable when compared to natural materials including decellularized matrices (Dong et al. 2008).

Natural material based scaffolds can either be constructed of natural biological components such as collagen, elastin, glycosaminoglycans (GAGs), fibrin, and hyaluronic acid or derived from decellularized autologous or xenogeneic tissues. Natural scaffolds exhibit several advantages over synthetic scaffolds. Due to their native ECM components, natural scaffolds elicit no apparent immunogenicity. Decellularized heart valves provide a scaffold that retains the native three-dimensional structure and extracellular matrix

components making them an ideal foundation for creating a successful TEHV (Dong et al. 2008).

The desired properties of natural scaffolds can be further enhanced through biochemical scaffold treatments. There has been work done to explore the effects and benefits of treatments to decellularized heart valve scaffolds to improve ECM degradation properties as well as increase cellular adherence during cell seeding. Through a process of dip coating or immersion in bioactive molecules such as fibronectin, a molecule containing an RGD peptide sequence, cell-matrix attachment can be enhanced. RGD peptides are one of the most effective and widely used peptides to promote cell adhesion (Dong et al. 2008). Cellular adhesion is a critical step in stable cell-matrix connections, providing physical support in early stage tissue construction (Sheng-Dong Huang et al. 2007). Any improvements in cellular adherence would prove invaluable to the cell seeding process as well as cellular retention under dynamic bioreactor conditioning.

As the main purpose of the decellularized scaffold is to provide a blueprint and native structure for seeded cells to adhere to as well as maintain ECM components and their underlying mechanical function, the ability to improve scaffold stability and degradation rates has been of interest to researchers. Once seeded and exposed to mechanical forces, cells begin to remodel existing ECM and laydown their own matrix relative to these mechanical stimuli. Improvement to degradation rates holds much potential for decellularized scaffold success. Glutaraldehyde-fixed bovine pericardium bioprosthetic heart valves have a good record of implantation in humans and have a long history of well-characterized mechanical and biological properties (Tedder et al. 2009).

Glutaraldehyde cross-linking fully stabilizes collagen however it does not allow for tissue remodeling and calcifies once implanted, both of which are non-ideal for a successful TEHV. Additionally, glutaraldehyde and similar crosslinking reagent are highly cytotoxic hindering their successful use for scaffold crosslinking in a living tissue engineered heart valve implant (Sung et al. 1998). Penta-galloyl glucose (PGG) is a collagen-binding polyphenol, which has shown promise as a moderate cross-linking agent for scaffold stabilization. PGG functions to interact with collagen thereby reversibly stabilizing acellular heart valve scaffolds. PGG cross-linking has been shown to be a non-toxic means for reversibly stabilizing scaffolds and controlling tissue degradation rates through cell mediated matrix remodeling (Tedder et al. 2009).

While glutaraldehyde cross-links collagen thereby protecting scaffolds from collagenase-enzymatic degradation, it has shown practically no stabilizing effect on either elastin or GAG components making glutaraldehyde treated scaffold highly vulnerable to both enzymatic elastin and GAG degradation. PGG, in addition to stabilizing collagen, also binds elastin and has also been shown to be efficient means of preventing elastase-mediated degradation (Chuang et al. 2009; Isenburg et al. 2004). The reversibly-stabilizing nature of PGG on both collagen and elastin matrix components of decellularized heart valve scaffolds makes PGG scaffold treatment an ideal means for retarding enzymatic scaffold degradation while also promoting a controlled remodeling by resident cells (Chuang et al. 2009; Sierad et al. 2010; Tedder et al. 2009).

1.7 Cell Seeding Techniques

In developing a device and method for seeding the surface of a tissue-engineered scaffold, there are several seeding techniques being researched. Cell seeding plays an integral role in production of a viable and successful implant. Especially in 3-D culturing, seeding is one of the most important steps greatly affecting cell growth and morphogenesis in the scaffold (Ouyang and Yang 2007). No matter what the targeted tissue is, the fundamental principle of combining a scaffold with cells lies at the foundation of tissue engineering. Literature indicates a number of different techniques for achieving cell seeding. However, there has been no seeding method deemed to be the gold standard.

Variances in scaffold shapes, structure, and compositions found throughout the tissue engineering field, necessitates differing methods for delivering cells to the scaffold. Though no one has demonstrated a correlation between the number of cells seeded and long-term implant function, optimization of the process is considered to be a large piece in the paradigm of tissue engineering (Villalona et al. 2010). Seeding techniques can be broken down into three categories static, dynamic, or perfusion seeding (Burg et al. 2000; Villalona et al. 2010).

1.7.1 Static Seeding

Static seeding, involves a means of seeding that doesn't utilize any form of motion to facilitate the application of cells to a scaffolds surface. The most common method of static seeding described involves manually pipetting a cellular suspension solution onto a scaffold surface and incubating the sample for a specified time (Figure 6)

(Villalona et al. 2010). As the seeding solution runs off the scaffold it can be re-pipetted onto the surface and incubated again and again (Burg et al. 2000; Villalona et al. 2010). Static seeding is the simplest and most widely used method of cell seeding. Requiring only a small volume of seeding solution and no complex mechanisms for its delivery, this method is inexpensive and strait forward in its application. Since this method relies on gravity, complex scaffolds such as heart valves make standardizing a uniform application of cells to the scaffold nearly impossible (Weinand et al. 2009).

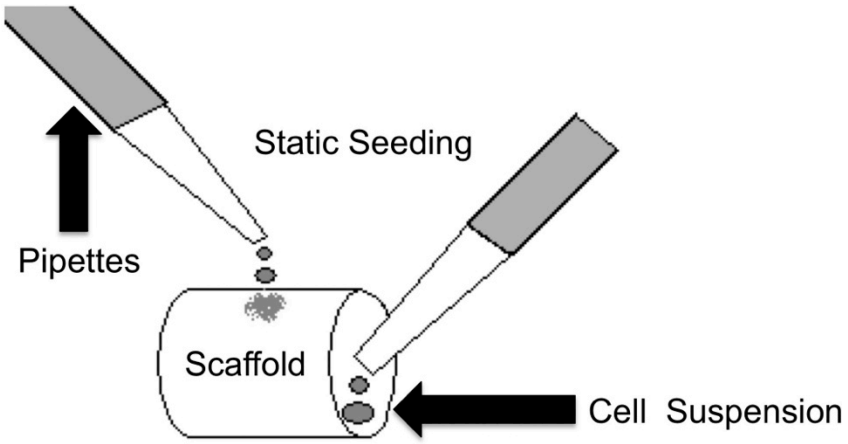


Figure 6: Statically Seeding Cells onto a Scaffold (Villalona et al. 2010)

The major drawback with static seeding is the very low seeding efficiencies achieved of 10-25% (Villalona et al. 2010). Another drawback comes with the fact that it requires manual pipetting and with it the introduction of human inconsistencies. Of which make achieving a uniform cell density difficult and often resulting in patchy cellular attachment on a scaffold surface (Weinand et al. 2009).

Though static seeding is the most common seeding method, with its low seeding efficiencies, the development of a successful dynamic seeding method capable of

increased efficiency and uniformity is in the interest of many research groups. The availability of cells surgically collected from a patient is often limited to small quantities so efficiency of cell use is very important (Weinand et al. 2009). There are a few different methods of dynamic seeding discussed in literature. While several of them focus on utilizing dynamic motion intended to maintain cells in suspension while in contact with the scaffold surface, other dynamic methods focused on the use of other forces such as magnetic, electrostatic, or perfusion to mate cells with the scaffold surface (Shimizu et al. 2007; Villalona et al. 2010; Griffon et al. 2011).

1.7.2 Dynamic Seeding

Dynamic seeding methods centered on the principle of fluid dynamics and motion of the cellular suspension solution with the purpose of maintaining cells in a state of constant suspension appeared to be more ideal for heart valve seeding due to the complex heart valve anatomy. Under dynamic conditions, high mixing intensity is a double-edged sword. It works to increase cell-surface contact frequency, which is critical for initial cell attachment. However, the high intensity mixing can also lead to the undesired result of cell detachment (Burg et al. 2000; Ouyang and Yang 2007).

One dynamic seeding method commonly used is the spinner flask (Burg et al. 2000; Ouyang and Yang 2007; Villalona et al. 2010). A simple design utilizes immersion where the scaffold is placed down into a volume of cell suspension solution while a stir bar agitates to maintain the cells in suspension (Figure 7). This method has virtually no precision in targeting specific surfaces of a scaffold for controlling cell to surface contact. The spinner flask requires a large volume of suspension solution as well as a large

number of cells to achieve relative cell seeding concentrations. This increased volume not only increases cost and waste but also reduces the random chance of cell-scaffold contact and subsequent adherence. In addition, the violent motion of the stir bar has an increased potential for damage and cell death. Though this method may work for some scaffolds, the randomness of cell to surface contact and inability to target or ensure contact with specific areas on the scaffold is the major drawback of the spinner flask in relation to seeding a heart valve. It greatly reduced the probability of cells contacting the targeted luminal surfaces making this method insufficient.

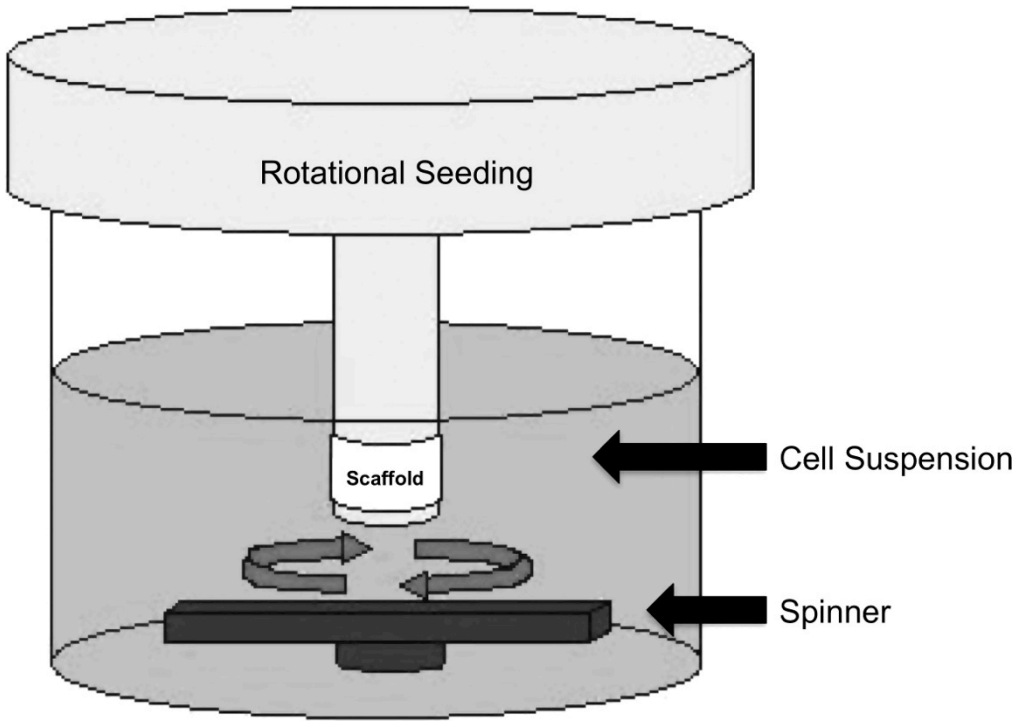


Figure 7: Dynamic Seeding of Cells on a Scaffold (Villalona et al. 2010)

Rotational Devices

Another dynamic seeding method discussed in literature uses rotation as a motive force to produce a randomized flow. This method provides a completely randomized fluid motion that is shown to be capable of maintaining cells in suspension thereby increasing the chance of cell to surface contact in all areas (Burg et al. 2000; Lueders et al. 2006; Arrigoni et al. 2008; Nieponice et al. 2008; Villalona et al. 2010; Griffon et al. 2011). Compared to the spinner flask, some rotational devices require a more difficult means of scaffold mounting. Additionally, they require a much smaller volume of seeding solution than the spinner flasks thereby reducing the number of cells needed to achieve high seeding solution concentrations and therefore reducing both cost and waste while improving cell-surface contact probability.

There are a few different designs in literature that use this type of motion for seeding. One design aiming to seed heart valves, mounts a scaffold in a chamber that in turn is secured inside an acrylic ball (Figure 8). The ball sits atop a base that is rotated via a pair of motors and wheels providing a randomized motion while maintaining suspension of cells (Lueders et al. 2006). A second style rotational device mounts the scaffold to a frame that fits inside a seeding chamber capable of a single plane of rotation (Figure 9). The frame is free to move around within the chamber providing the random cell to surface contact as well as mixing of the cells around the scaffold (Gulbins et al. 2005).

Rotational dynamic seeding methods have been shown to successfully function in seeding cells onto the complex anatomic structure of heart valves (Gulbins et al. 2005)

(Lueders et al. 2006). When compared to other methods, rotational devices provide the most efficient targeted application of cells to these complex structures while promoting randomized adherence to all surfaces.

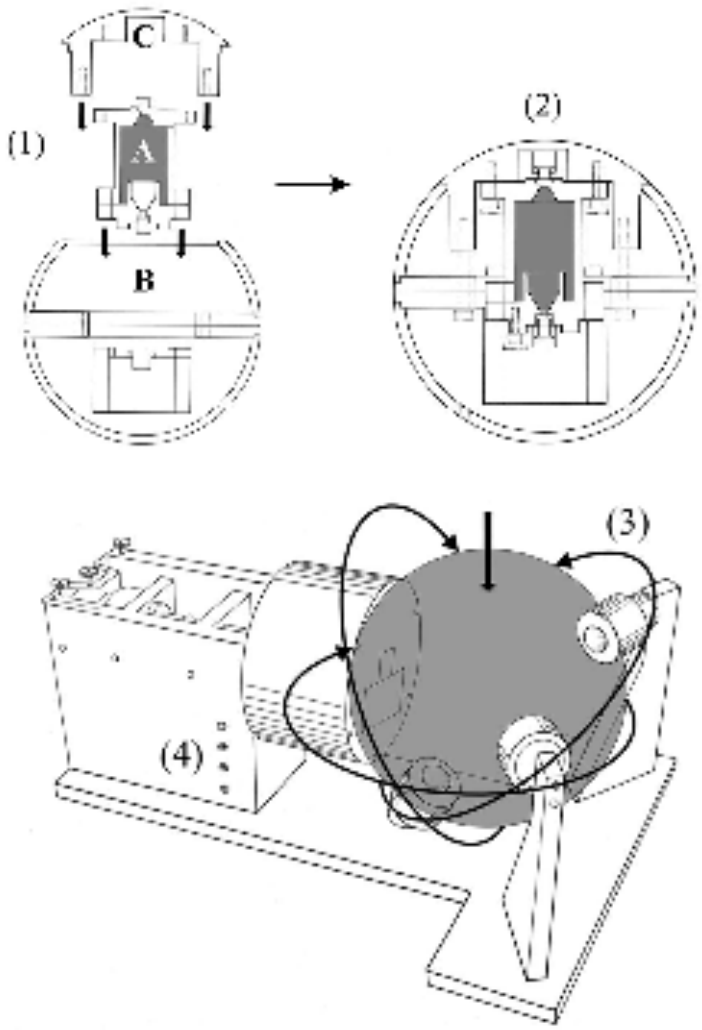


Figure 8: Acrylic Ball Dynamic Rotational Device (Lueders et al. 2006)

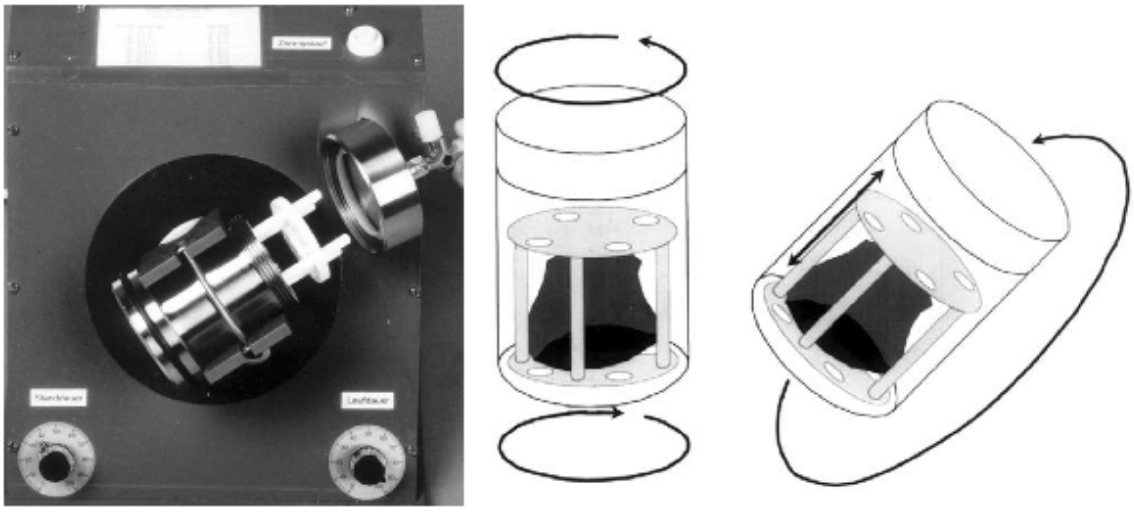


Figure 9: Free Floating Mount Rotational Seeding Device (Gulbins et al. 2005)

1.7.3 Perfusion Seeding

Perfusion seeding methods rely on either magnetic, hydrostatic pressure, or electrostatic forces to drive cells onto or into a scaffold (Burg et al. 2000; Shimizu et al. 2007; Villalona et al. 2010; Arrigoni et al. 2008; Griffon et al. 2011). This seeding method would be difficult to implore on a heart valve scaffold due to complex geometry and fluid dynamics of the valve. A pressure gradient can be used to force seeding solutions into a scaffold by increasing internal pressures or pulling an external vacuum (Figure 10). A second means for achieving the necessary forces to promote cell-surface contact uses the power of magnets (Figure 11). Through the use of magnetic nanoparticles bound to cell surfaces, the cells can then be manipulated through precise use of magnets to direct cells onto specific surfaces of a scaffold (Shimizu et al. 2007; Villalona et al. 2010). Through a third method utilizing electrostatic forces, cell seeding is performed by inducing a positive surface charge on a scaffold, which attracts negatively charged cells onto the scaffold (Villalona et al. 2010).

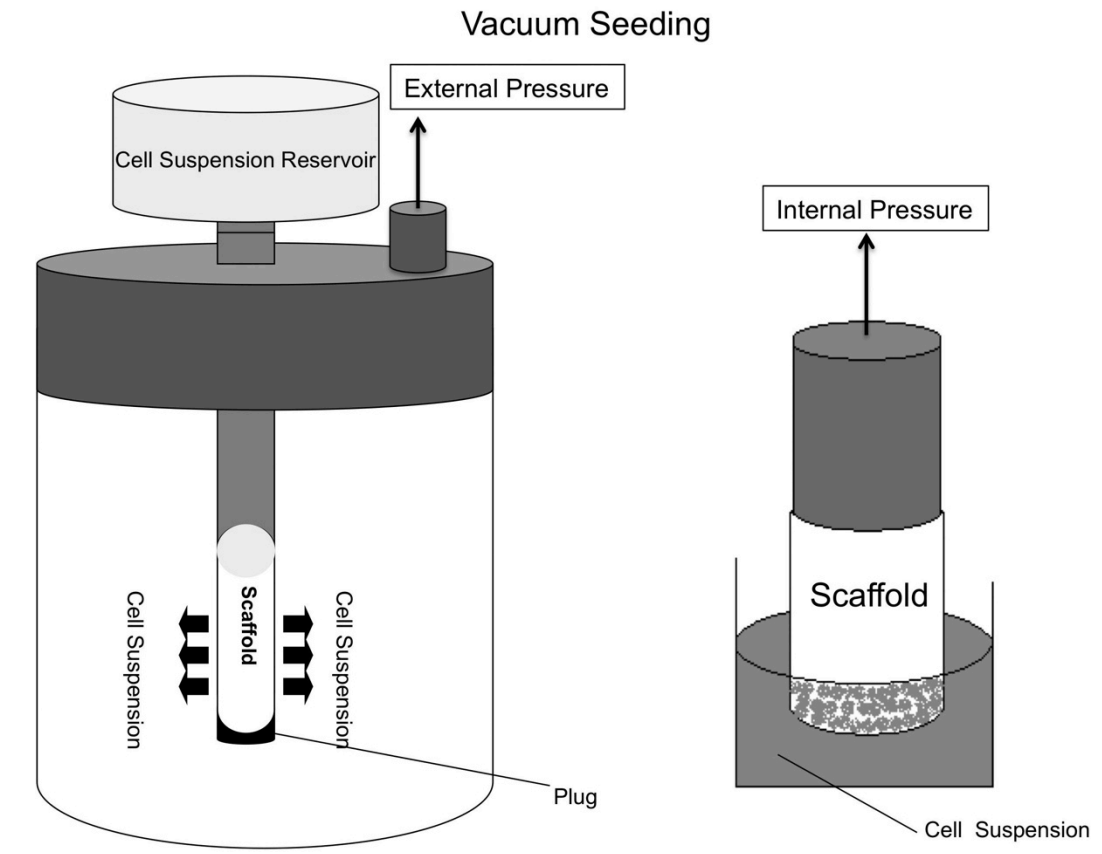


Figure 10: Perfusion Cell Seeding Example (Villalona et al. 2010)**.**

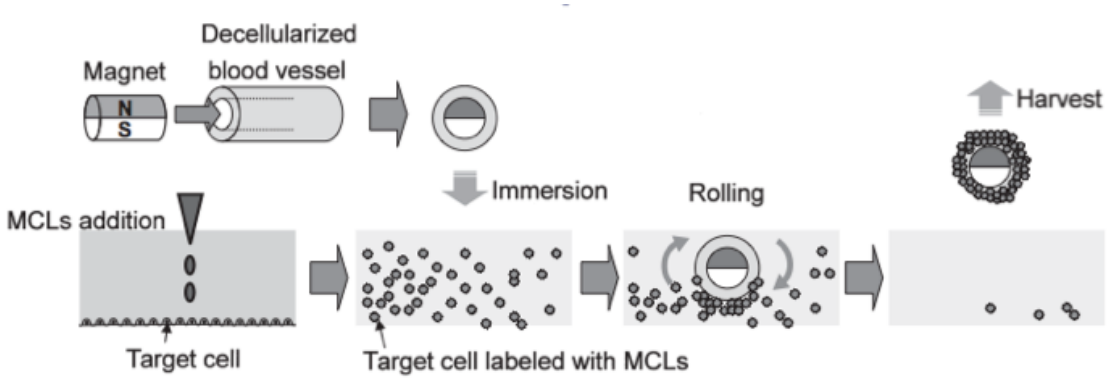


Figure 11: Magnetic Cell Seeding (Shimizu et al. 2007)**.**

With each method comes both advantages and disadvantages making them better suited for a specific scaffold and tissue engineering application. Studying these various

cell-seeding methods offers insight into the design of an optimized dynamic heart valve seeding device.

CHAPTER TWO

PROJECT APPROACH AND RATIONALE

The goal of my master's thesis research was to develop a device and method for seeding cells in a confluent layer onto the surface of a decellularized PGG-stabilized porcine aortic scaffold developed within our lab.

Previous seeding attempts have focused on a static cell seeding method whereby a cell suspension was repeatedly pipetted on the surface of the heart valve scaffold. As the cellular suspension solution ran off of the scaffold it was re-pipetted onto the surface (Sierad et al. 2010). This static seeding method resulted in successful surface adherence of cells though these results were far from achieving the confluent endothelial layer necessary for development of a viable implant. These shortcomings ultimately defined **the need** for my master's thesis project.

The process of developing a device and method began with an **overview of the requirements and strategy** for meeting our need and achieving the goal.

After reviewing the methods in literature used by others for dynamic seeding and specifically targeting the needs of our lab's application, the following **Project Aims** were established:

- To achieve a more uniform cellular attachment than that observed via static seeding methodologies.
- To progress toward achieving a confluent valvular endothelium.
- To investigate cellular retention under dynamic bioreactor conditioning.
- To maintain sterility throughout seeding and bioreactor testing.

In moving toward achieving these aims, several **Design Parameters** were defined:

- Simplify design

- Maintain sterility
- Provide a physiologic environment
- Maximize cell-seeding efficiency
- Minimize cost
- Utilize existing lab equipment

MATERIALS AND METHODS

3.1 Scaffold Preparation

3.1.1 Fresh Tissue Harvesting

A local USDA approved abattoir, Snow Creek Meat Processing in Seneca, SC, generously provided access to fresh porcine hearts. The hearts were dissected at the processing facility to remove the aortic heart valve and sufficient surrounding tissue to enable proper mounting in the dynamic seeding device. In addition to the aortic valve itself, the attached cardiac muscle, and approximately two inches of the aortic root were place in 500 milliliters of double distilled water (ddH_2O). The 500 milliliter containers were kept on ice and transported back to Clemson University's Biocompatibility & Tissue Regeneration Lab (BTRL). Back in the lab, the valves were further trimmed and cleaned over ice. Fat was excised from the surface of the valves and the aorta was trimmed to a height of 1.5 inches above the valves. The attached muscle tissue was trimmed down to a thickness of 1-3 millimeters and radial width of 1 inch beyond the aorta. Once clean, the valves were ready for decellularization (Figure 12 &Figure 13).

3.1.2 Heart Valve Decellularization

Utilizing an established decellularization protocol developed previously in our lab, the porcine aortic heart valves were subjected to a series of chemical washes and hypotonic ddH_2O rinses. In order to minimize the host response post implantation, the removal of the native porcine cells and associated antigen Gal-α was crucial in in the development of a viable tissue engineered heart valve scaffold. Cellular components and fragments were removed with a sodium hydroxide (0.05 NaOH) wash followed by an

overnight incubation in a decellularization solution composed of sodium dodecyl sulfate (0.05% SDS), Triton X-100 (0.5%), deoxycholic acid, sodium Salt (0.2%), and ethylenediaminetetra acetic acid (0.2% EDTA). Finally the valves were washed in a RNase/DNase solution to remove any remaining porcine deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) present in the tissue. Following decellularization, the valves underwent a sterilization step, rinsing in 0.1% peracetic acid for 2 hours. With the valve now decellularized and sterile, the valves were ready for chemical fixation with a 0.075% PGG solution (Figure 12 & Figure 13). See Appendix A**: (6.1.1)**

3.1.3 Fixation

In hopes of maintaining both the structure and mechanical properties of the decellularized tissue, the valves were subjected to a chemical crosslinking treatment. Under sterile conditions, the valve cusps were packed in a closed position using penta-galloyl glucose (PGG) soaked cotton balls. Packed cusps underwent an overnight incubation in a 0.75% PGG solution (Figure 12 & Figure 13). See Appendix A**: (6.1.2)**

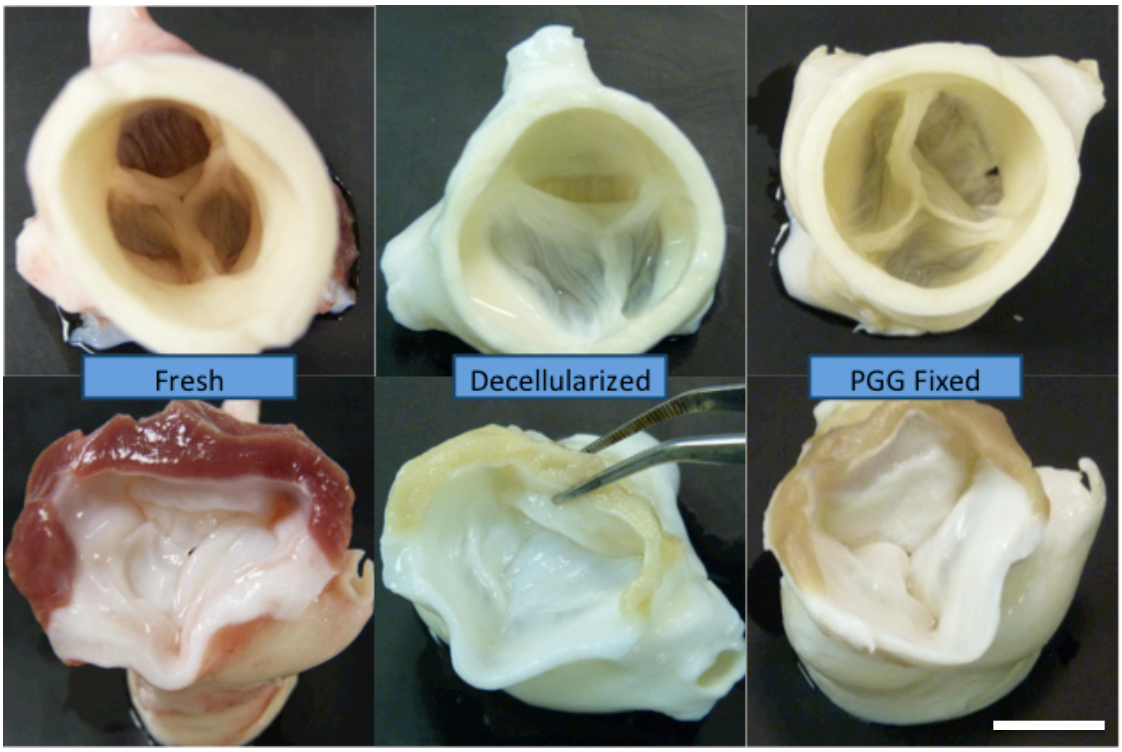


Figure 12: Porcine Aortic Heart Valve Root. Steps Through Scaffold Preparation Protocol. Top Row- View From the Aorta (Fibrosa Surface) . Bottom Row- View From the Ventricle (Ventricularis Surface) Bar=1cm

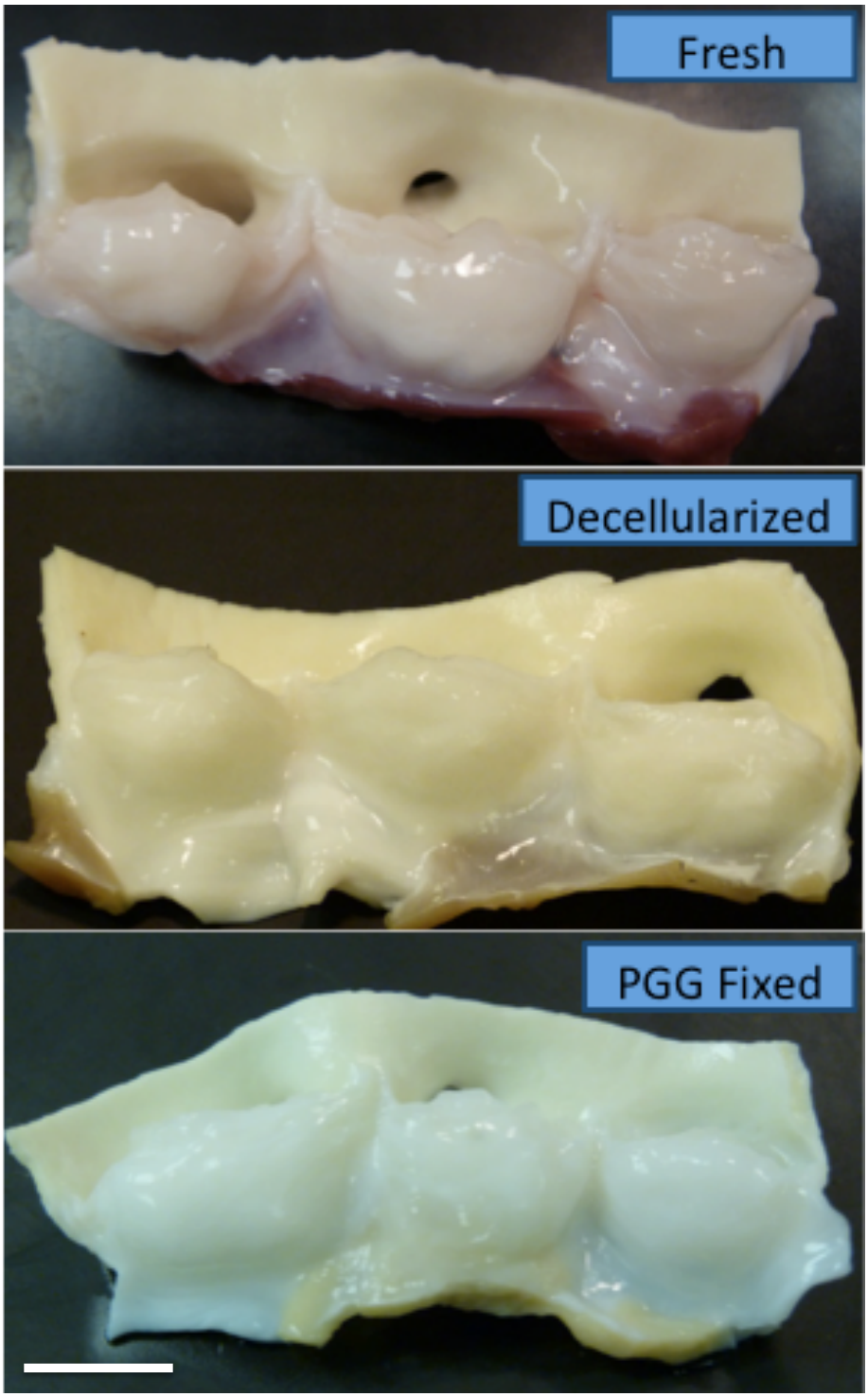


Figure 13: View of Aortic Heart Valve After Opening by Dissection. Steps Through Scaffold Preparation Protocol. Bar=1cm

3.2 Porcine Aortic Endothelial Cell Culture

Porcine Aortic Endothelial Cells were sourced from Cell Applications (Cell Applications Inc., San Diego, CA) and plated on cell culture flasks coated with fibronectin with the desired concentration of 1 $\mu g/cm^2$ to promote cellular attachment. The medium used was MCDB 131 (Mediatech, Manassas, VA), 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 1% Antibiotic-Antimycotic (Ab/Am) Solution (Mediatech, Manassas, VA). When cell density in the culture flask reached about 80% confluence, the cells were passaged and counted using Millipore's Scepter ^{TM} cell counter. Cells were then expanded further into additional T-150 culture flask until desired cell numbers were obtained for seeding.

3.2.1 Fibronectin (FN) Cell Culture Flask Coating Protocol

Cell culture flasks were coated with fibronectin before cells were passaged in and plated onto flasks. Following the protocol found in Appendix B**: (6.2.1)**

3.2.2 Cell Culture and Confluent Cell Passaging

Once cells had been expanded to ~80% confluency in the culture flasks, the cells were trypsinzed and passaged into additional flasks (Figure 14). The trypsinized cells were spun down in a centrifuge and the supernatant removed from the cell pellet. After resuspending the pellet the cells were counted using the Milipore Sceptor^{TM} and the protocol found in Appendix B**: (6.2.3)**. The cells were split into fibronectin coated flasks and placed back in the incubator. See Confluent Cell Passaging Protocol in **Appendix B: (4.8.2).**

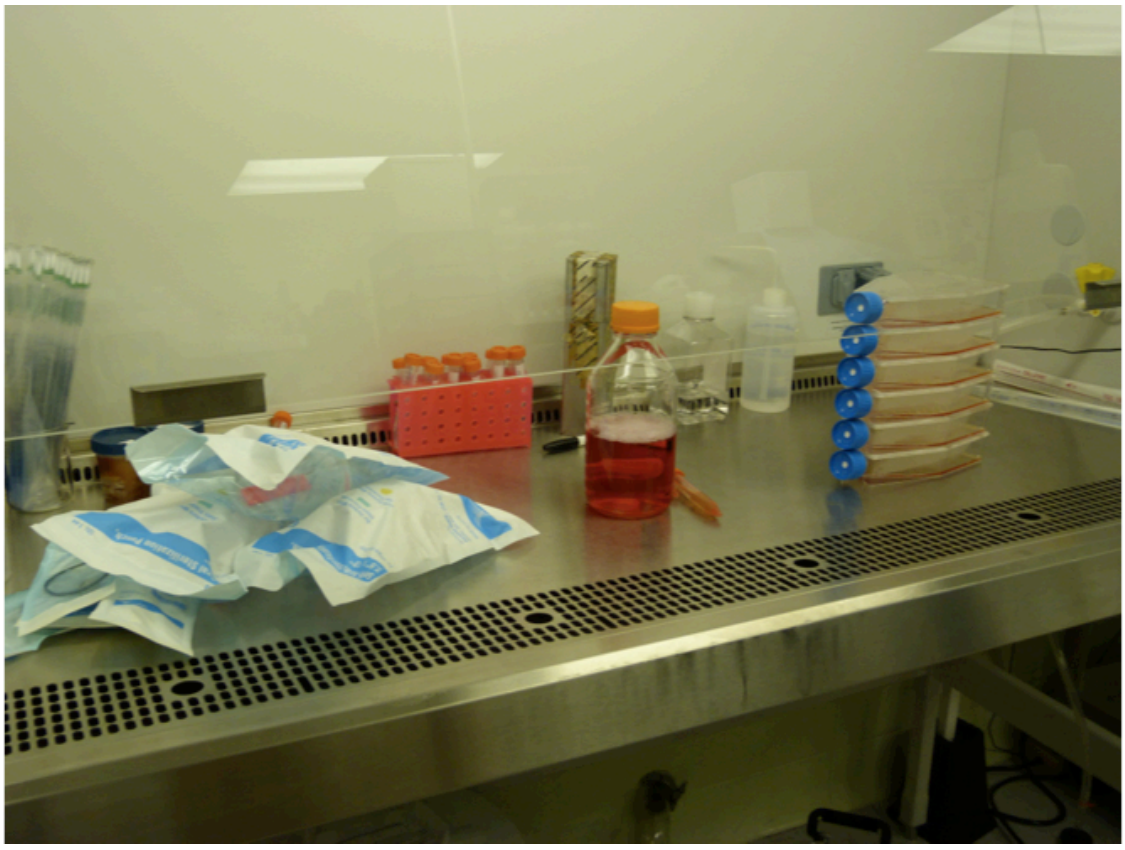


Figure 14: Porcine Aortic Endothelial Cell Culture. From Left to Right, Sterilized Cell Seeding Chamber, Cell Culture Medium, Trypsin, T-150 Culture Flasks with ECs

3.2.3 Cell Counting

Cell counting was performed using the Milipore Sceptor. Following the manufacturer supplied protocol. See Appendix B**: (6.2.3)**

3.3 Dynamic Seeding Device Design

The physical design process centered on maintaining simplicity. Working constantly to keep this in mind the seeding device was to utilize as few moving parts as necessary and the design process started at the foundation, the scaffold, and worked outward. In addition, the design process took into account basic physics incorporating constants such as the force of gravity and the understanding that cells would naturally fall

out of suspension when not under dynamic forces. The complex anatomy of the heart valve, mainly the cusps and their functional purpose of maintaining unidirectional flow was carefully taken into consideration at this initial stage of design.

Previous researchers in the BTRL developed a means for mounting valves in a purpose designed heart valve bioreactor utilizing a method of sandwiching tissue surrounding the valve between two mounting rings (Figure 15 &Figure 16). This method was found to allow the valve to function in the bioreactor just as it would naturally in the heart. Keeping this mounting method in mind, the integration of the cell seeding step into the overall tissue engineered heart valve development procedure could be achieved in a smoother and more timely manner.

Bridging this transition between the seeding and conditioning steps is important in minimizing the handling of the valve, critical for maintaining sterility, and subsequently a leading design factor for maintaining the bioreactor mounting method. The unidirectional flow of the heart valve warranted a slight modification of the bioreactor mounting rings to allow the fluid to flow around the valve and contact all surfaces. Slits were cut through the mounting rings to achieve this. After establishing this method of mounting, the next step in design was to create the actual seeding chamber (Figure 17).

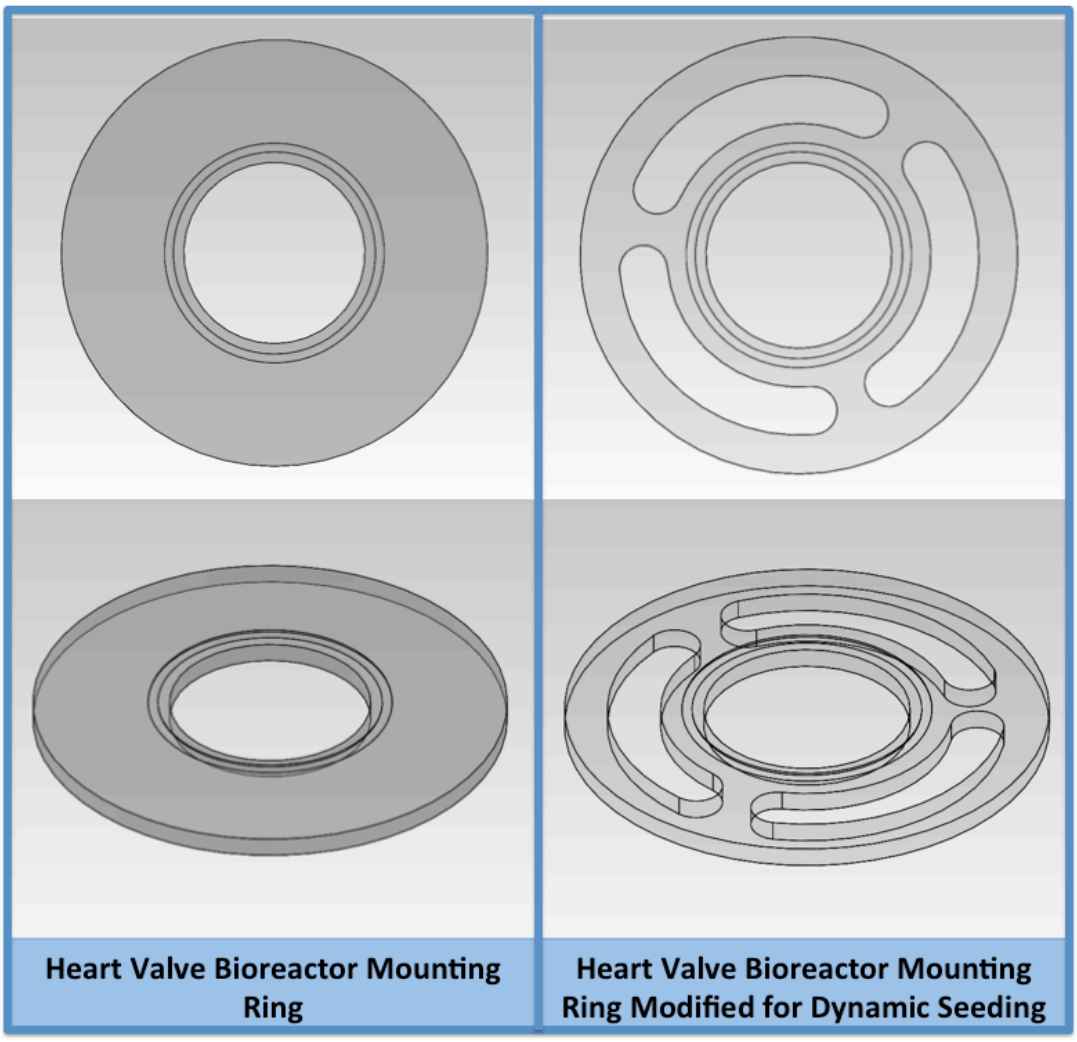


Figure 15: Heart Valve Bioreactor Mounting Ring and Modifications for Dynamic Seeding Device. Dimensions=Appendix C

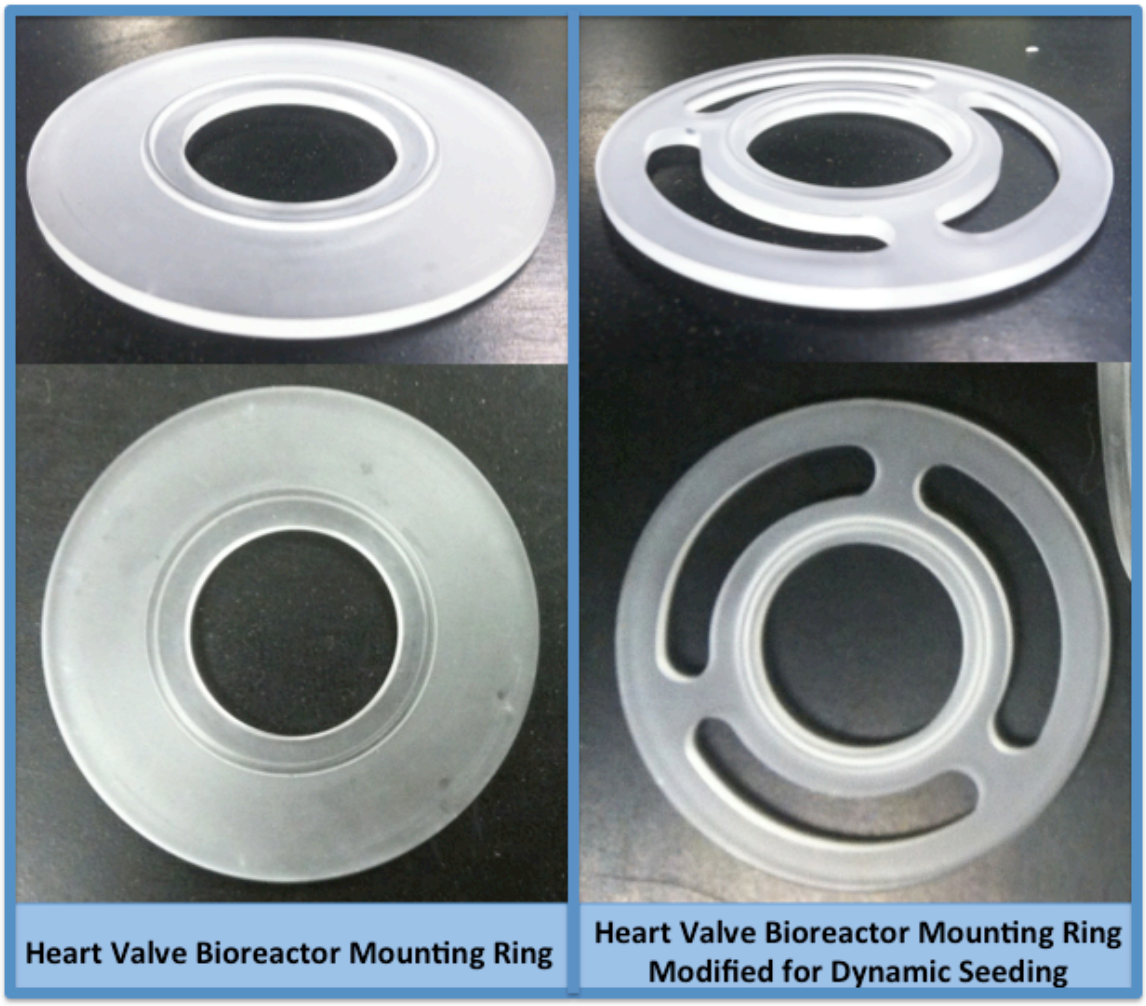


Figure 16: Heart Valve Bioreactor Mounting Ring and Modifications for Dynamic Seeding Device. Dimensions=Appendix C

Taking into consideration the dynamic seeding devices in literature and improving upon them, the design approach aimed to reduce the amount of seeding solution required so cell concentration and subsequently surface contact could be maximized. This approach would minimize cost as well as waste of cells and unnecessary seeding solution. In achieving this optimized design, the seeding camber was to follow the basic shape of a valve and attached aorta while maintaining a slightly larger diameter. The chamber's

edges were rounded for the purpose of accomplishing a smooth non-violent flow of the cell seeding suspension around the valve. The edges of the chamber were designed to align with slits in the rings to further the purpose of reducing violent flow that could lead to cell death. The lid was designed to slide into the seeding chamber and pinch the mounting rings thereby holding the valve in place (Figure 18). The inside edge of the lid was designed with a curve that also aligned with the slits in the mounting rings giving the chamber and lid inside surfaces a continuous flow around the valve. In order to maintain a sterile seal between lid and seeding chamber, an O-ring was incorporated into the design.

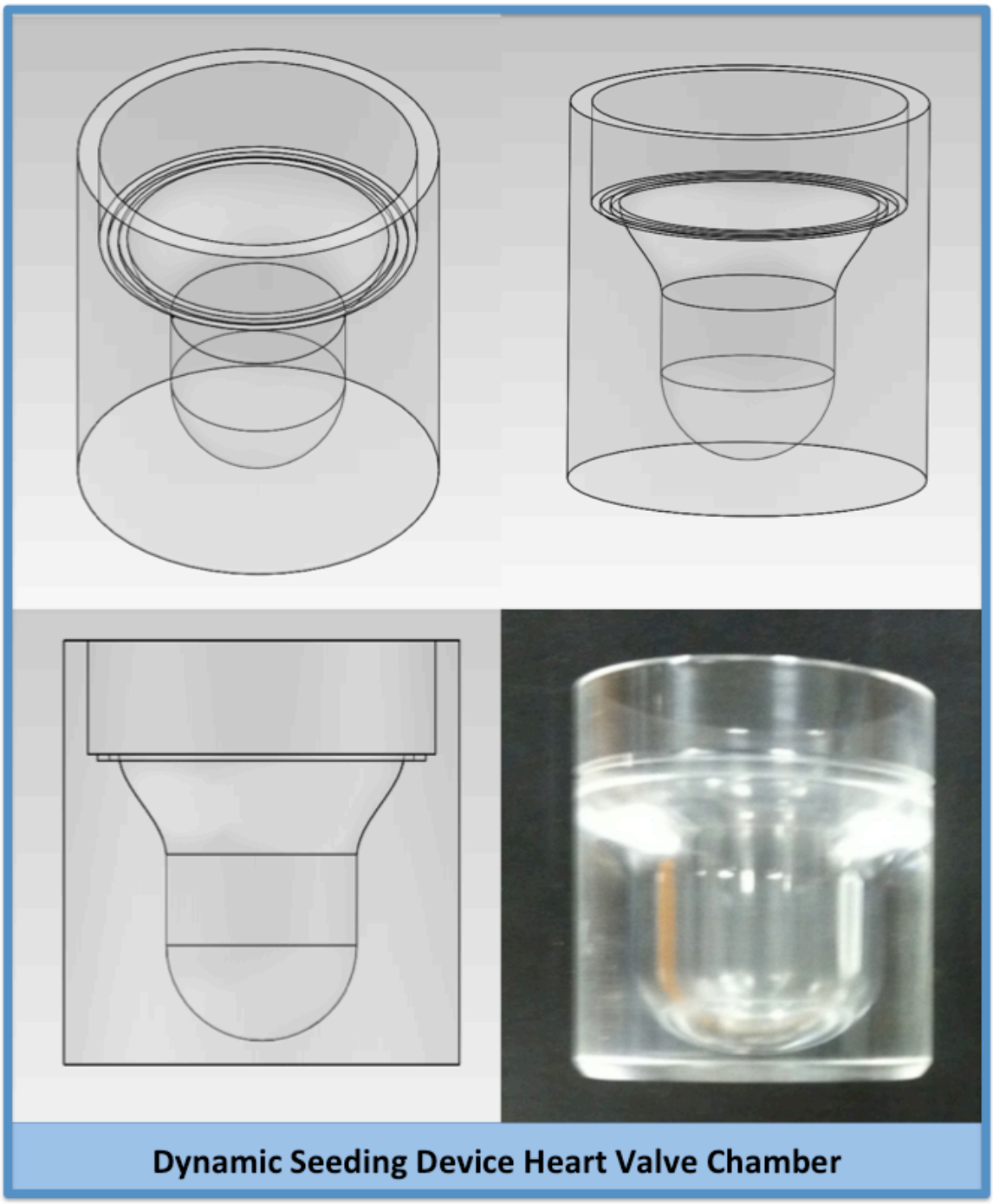


Figure 17: Dynamic Seeding Device Chamber. Dimensions=Appendix C

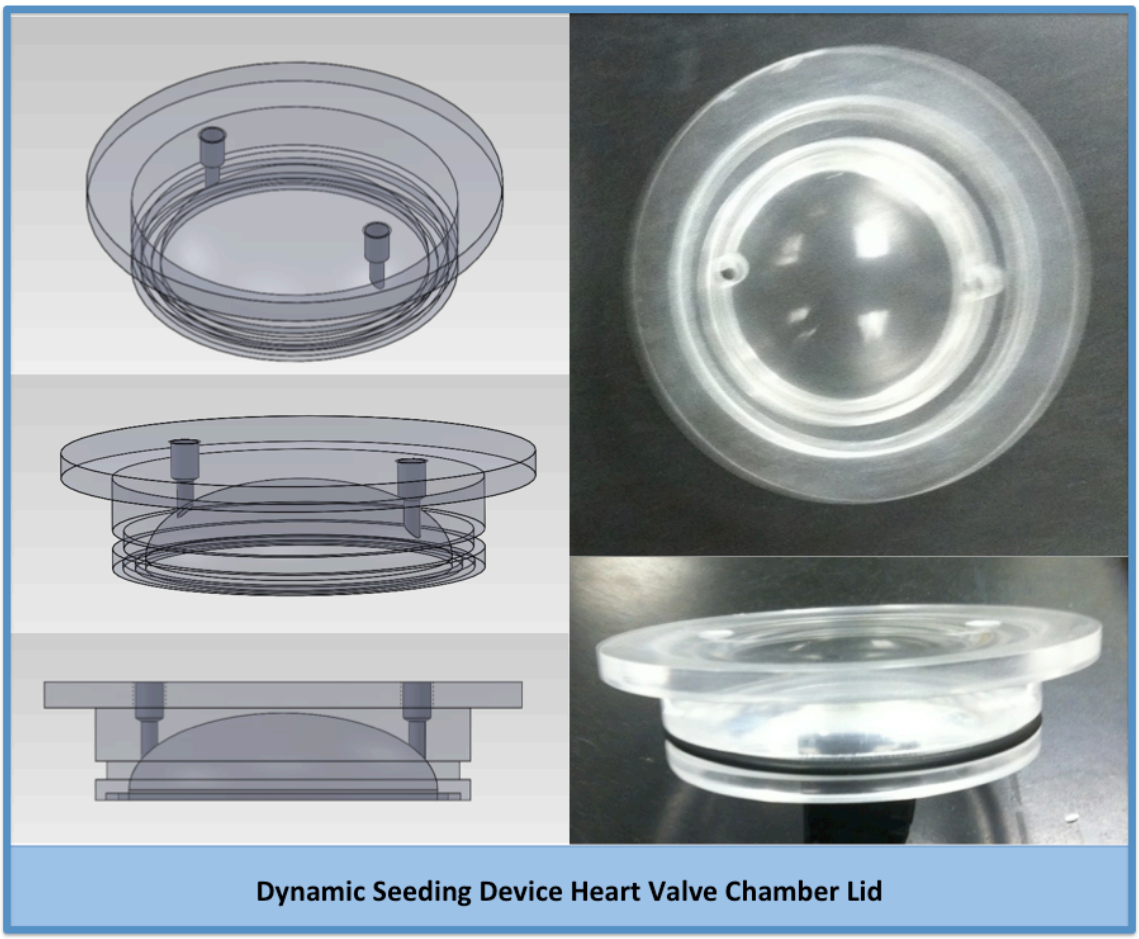


Figure 18: Dynamic Seeding Device Heart Valve Chamber Lid. Dimensions=Appendix C

To gain access to the chamber with its sterile seal, the necessity for a means to relieve pressure and vacuum was addressed through the incorporation of two ports found in the lid. The ports were machined with a threaded ¼ inch hole making them capable of accepting a Luer lock fitting and plug. These access ports serve a number of purposes. They allow for equalization of pressure between the chamber and the outside atmosphere. They also provide a means to access the chamber for adding or removing solutions with the lid on as well as the ability to attach a sterile filter with great ease to facilitate sterile gas exchange to the heart valve seeding chamber.

Now that the design had been conceptualized, the all-important parameter, when working with cells and living tissues, of maintaining sterility was addressed. Without maintenance of sterility, all else is a failure. Addressing this, the selection of a material and its ease of sterilization were investigated. Also playing a major role in material selection was material and machining cost. Acrylic was the chosen material as it was easy to render sterile through ethylene oxide treatment. An added benefit of acrylic to cost and sterility is its translucent properties allowing for visual observation of what was happening within while seeding is underway. Through the use of ethylene oxide and steam sterilization all parts of the device could easily be rendered sterile prior to any cell seeding study.

Another important design parameter was the ability to maintain physiologic conditions. In addressing this need, it was determined that the device should be small enough to fit inside a bench top incubator that was already owned by the lab thereby also reducing overall cost.

Of most importance was the determination of the type of motion that the valve should undergo during the cell seeding process. The dynamic motion of the device determines the flow of suspension solution, the cell to surface contact, and ultimately the uniformity and confluency of cellular attachment on the valve surface. A three-dimensional motion was decided on, combining an end over end rotation with an orbital shaking motion. The end over end flow of the device is ideal for ensuring cells flow through the valve scaffold and come in contact with the interior surface of the valve and its cusps (Figure 20). The slow speed orbital shaking gives the solution another axis of

motion and its incorporation works to maintain the suspension of the cells in solution. The orbital motion was achieved utilizing an orbital shaker already owned and in use by the BTRL. This also reduced cost by eliminating the need to purchase additional equipment. Combined, these motions would have to be studied in their ability to achieve a random motion with the aim of targeting all surfaces of the valve for cellular attachment.

Having the design parameters defined, the device was drawn up using SolidWorks drafting software. The Clemson University Machining and Technical Services located on campus was tasked with machining the acrylic along with the frame for mounting the device to the bench top shaker. Utilizing the SolidWorks files the machinist milled the acrylic and aluminum frame to exact specifications (Figure 19-Figure 23). See Appendix C for SolidWorks Drafts.

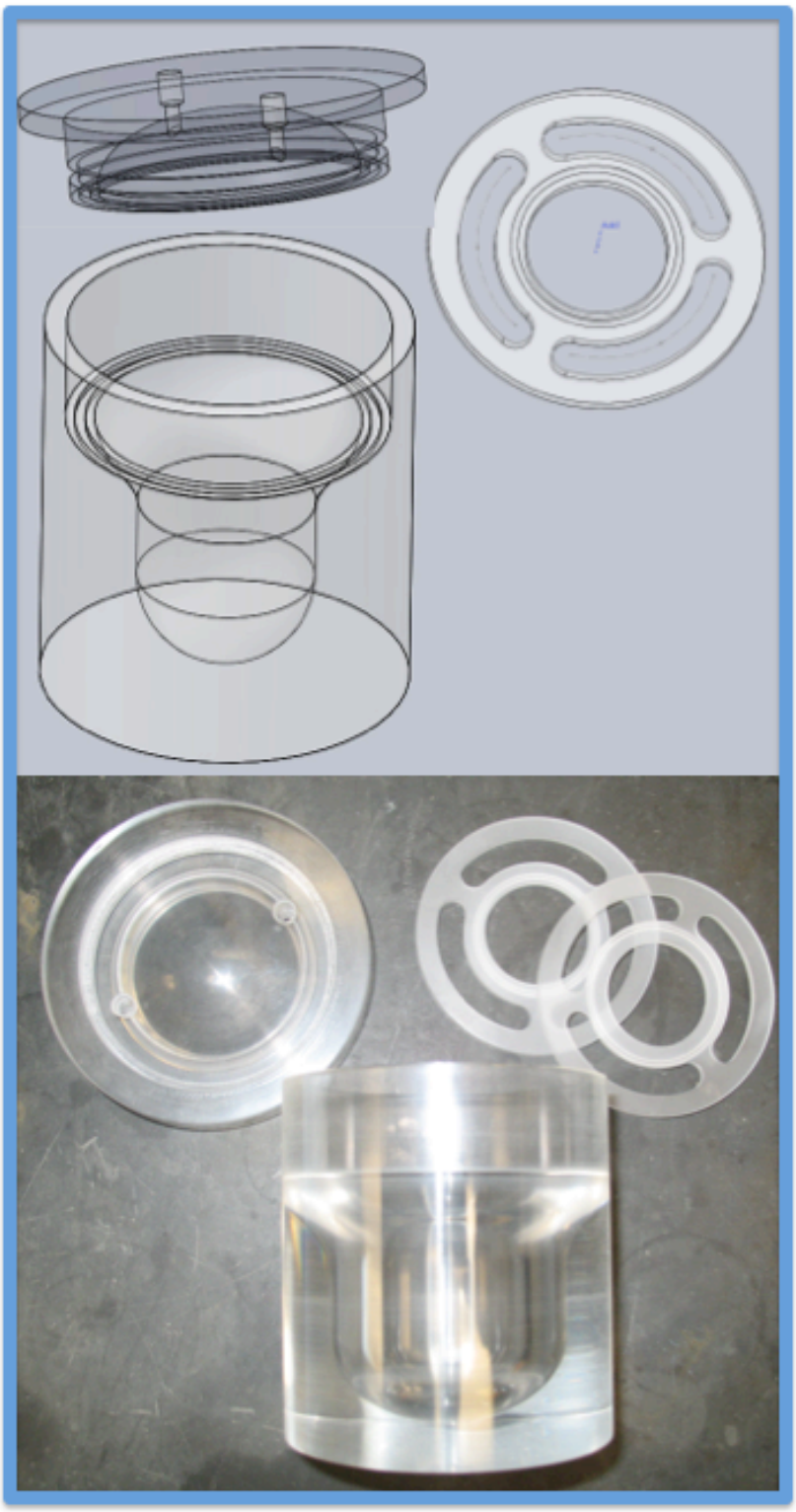


Figure 19: Acrylic Dynamic Cell Seeding Chamber. Dimensions=Appendix C

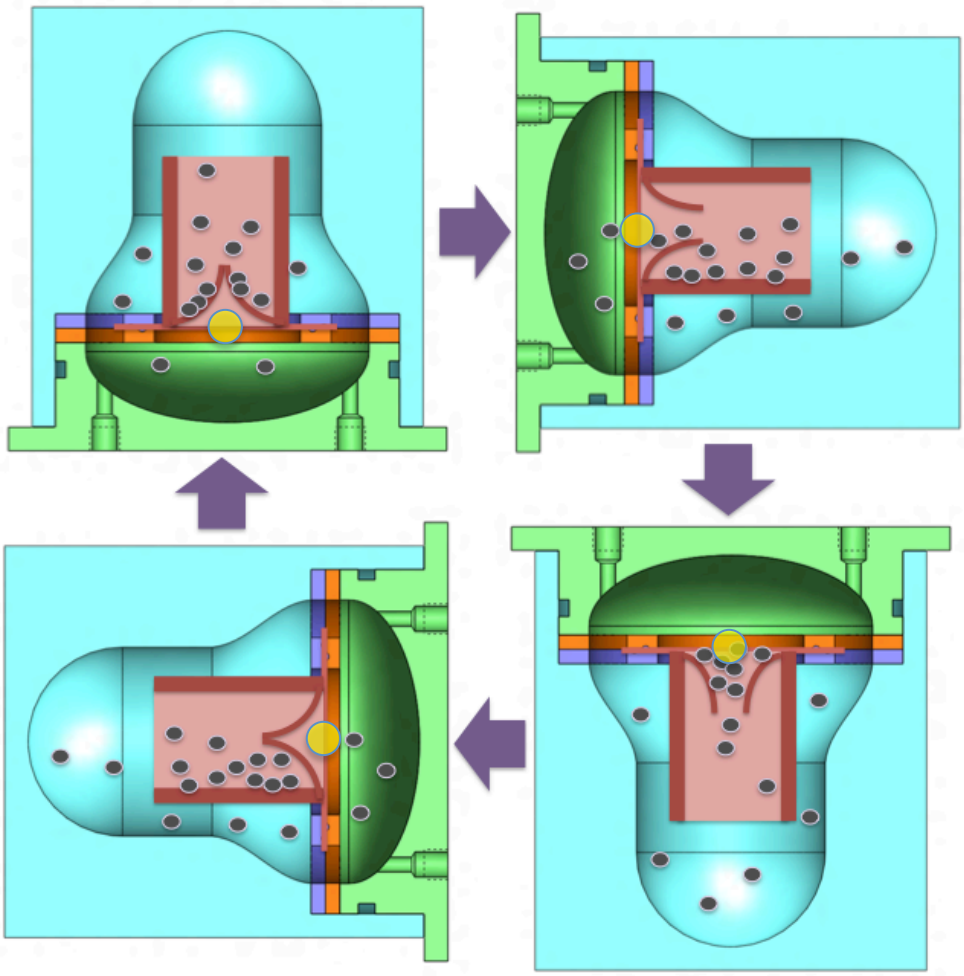


Figure 20: Designed Flow of Cells Through the Heart Valve Scaffold. Blue=Seeding Chamber, Green=Lid, Orange & Purple=Mounting Rings, Red=HV Scaffold, Black=Endothelial Cells, Yellow=Axis of Rotation

After the first three studies described below, additional design modifications and improvements were taken into consideration. After preliminary test of the device rotated by hand and proof of concept, the machine shop turned to helping with the addition of a stepper motor, microprocessor, and National Instruments' LabView Software necessary for control of the motion, speed, and time intervals between varied rotational directions. The design upgrades are described in Study 4 (3.4.6).

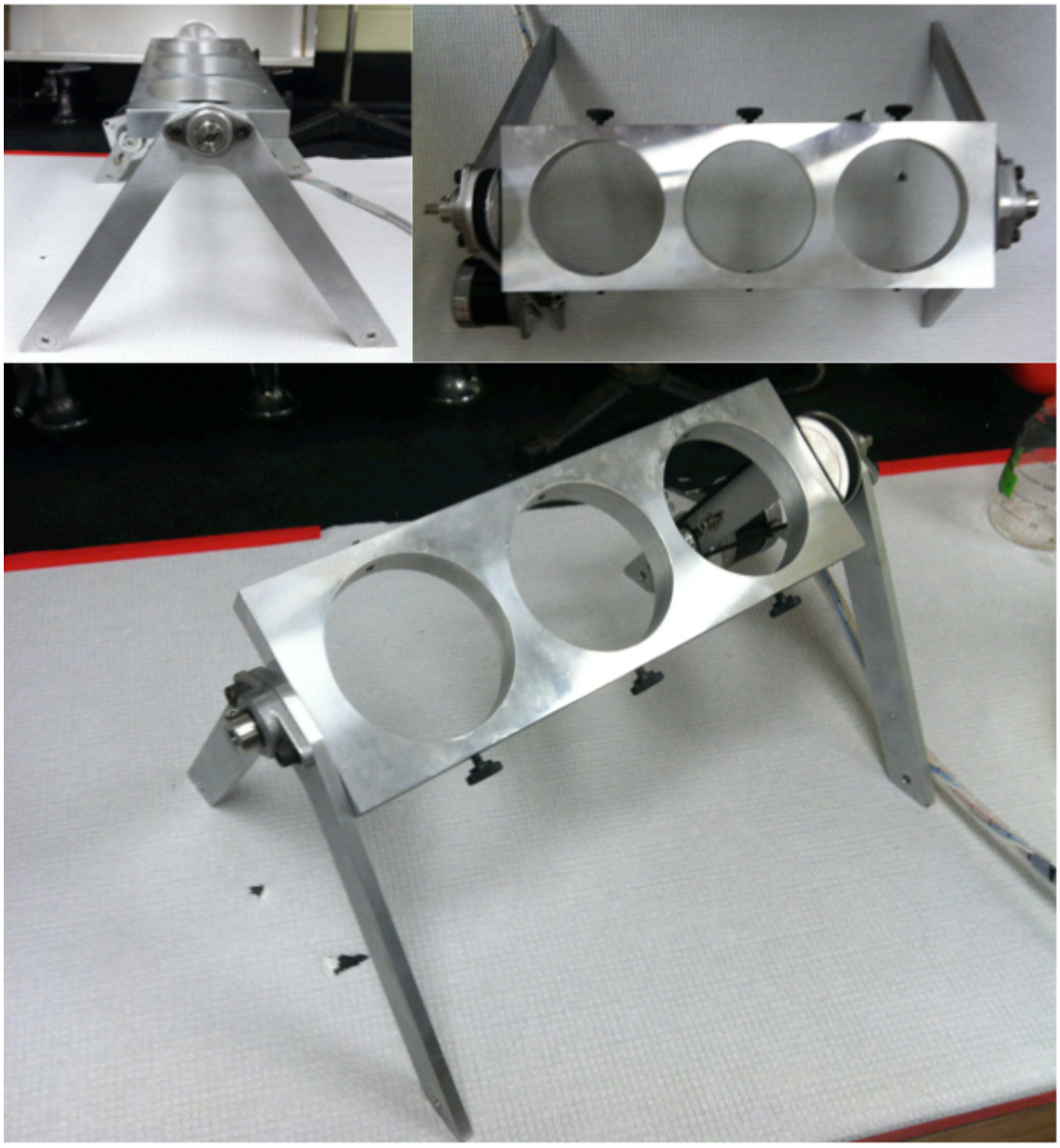


Figure 21: Aluminum Seeding Chamber Mounting A-Frame

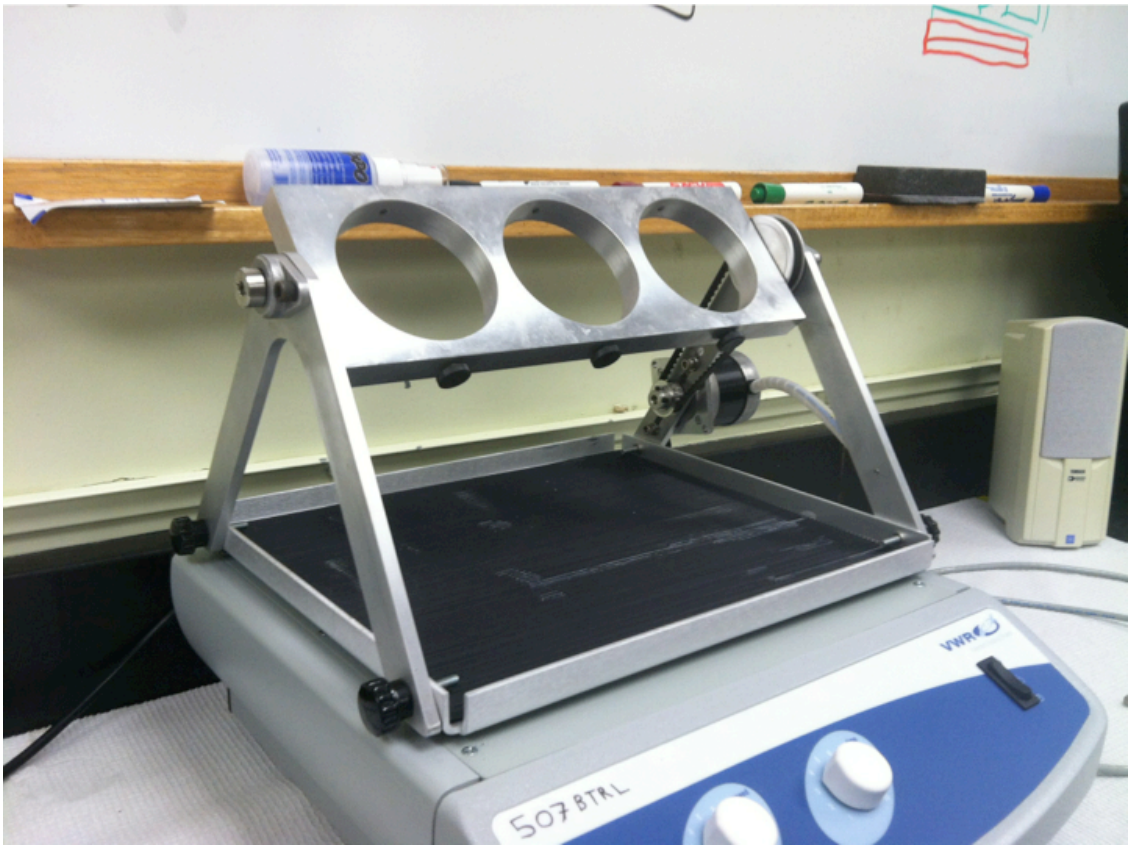


Figure 22: Aluminum Shaker Frame and Stepper Motor

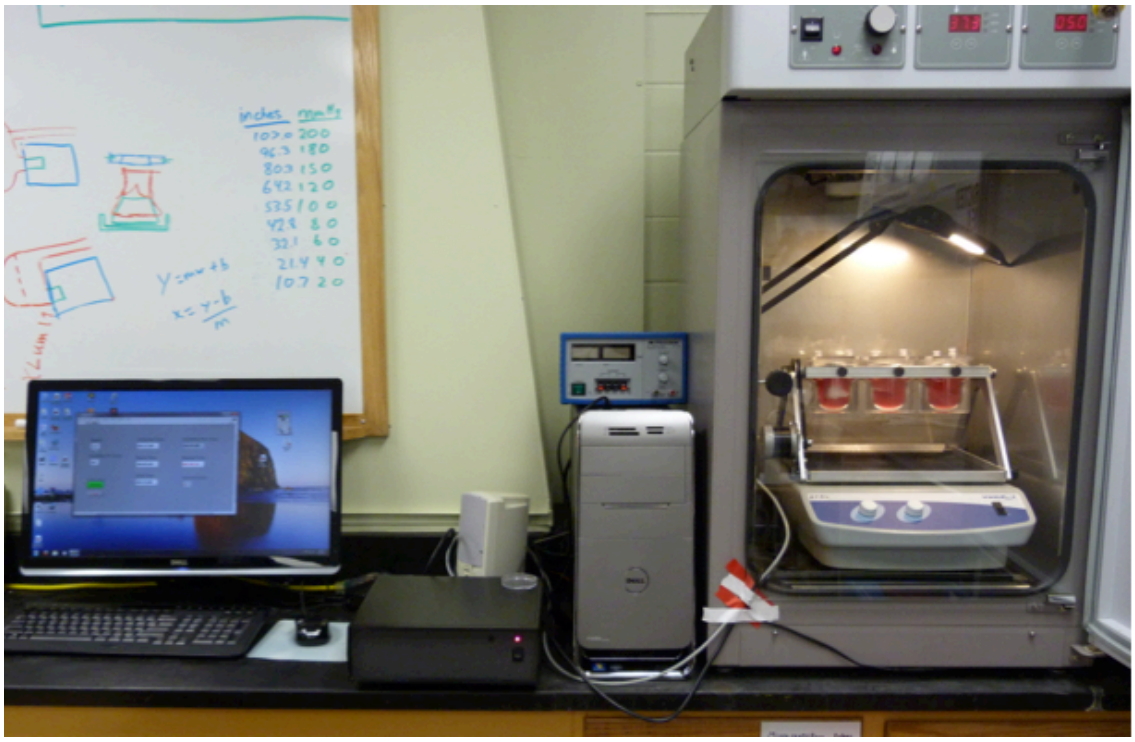


Figure 23: Overview of Device in Incubator with Computer Control

3.4 Porcine Aortic Endothelial Cell Seeding Studies with Dynamic Seeding Device

After the design process several studies were iteratively developed to investigate and optimize cell-seeding procedure.

Table 1: Overview of Studies

Overview of Studies	
Study 1	Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study)
Study 2	Initial Low Concentration Cell-Seeding Study
Study 3	Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity
Study 4	Computer Control for Increased Coverage and Variable Seeding-Time Study
Study 5	Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions
Study 6	Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions

3.4.1 Study 1: Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study)

In designing a series of studies with the goal of developing a protocol for optimizing the cell seeding effectiveness with our dynamic seeding device, there were several parameters that first needed to be established. Study 1's goal was to investigate the dynamic seeding device's ability to maintain cells in suspension. In addition it would provide initial data with respect to speed, fluid dynamics, and axis of rotation (Figure 24). A decellularized PGG stabilized porcine aortic heart valve was mounted in the device and

the chamber filled with water mimicking seeding solution and black pepper representing cells. Black pepper was chosen due to its low cost and low density making it fall out of suspension at a slower rate as well as its ability to be easily seen (Figure 26). Rotating the device at slow speed by hand the movement of the pepper and fluid flow could be observed (Figure 25).

Figure 24: Study 1: Defining an Axis of Rotation. Looking at Flow Around the Valve

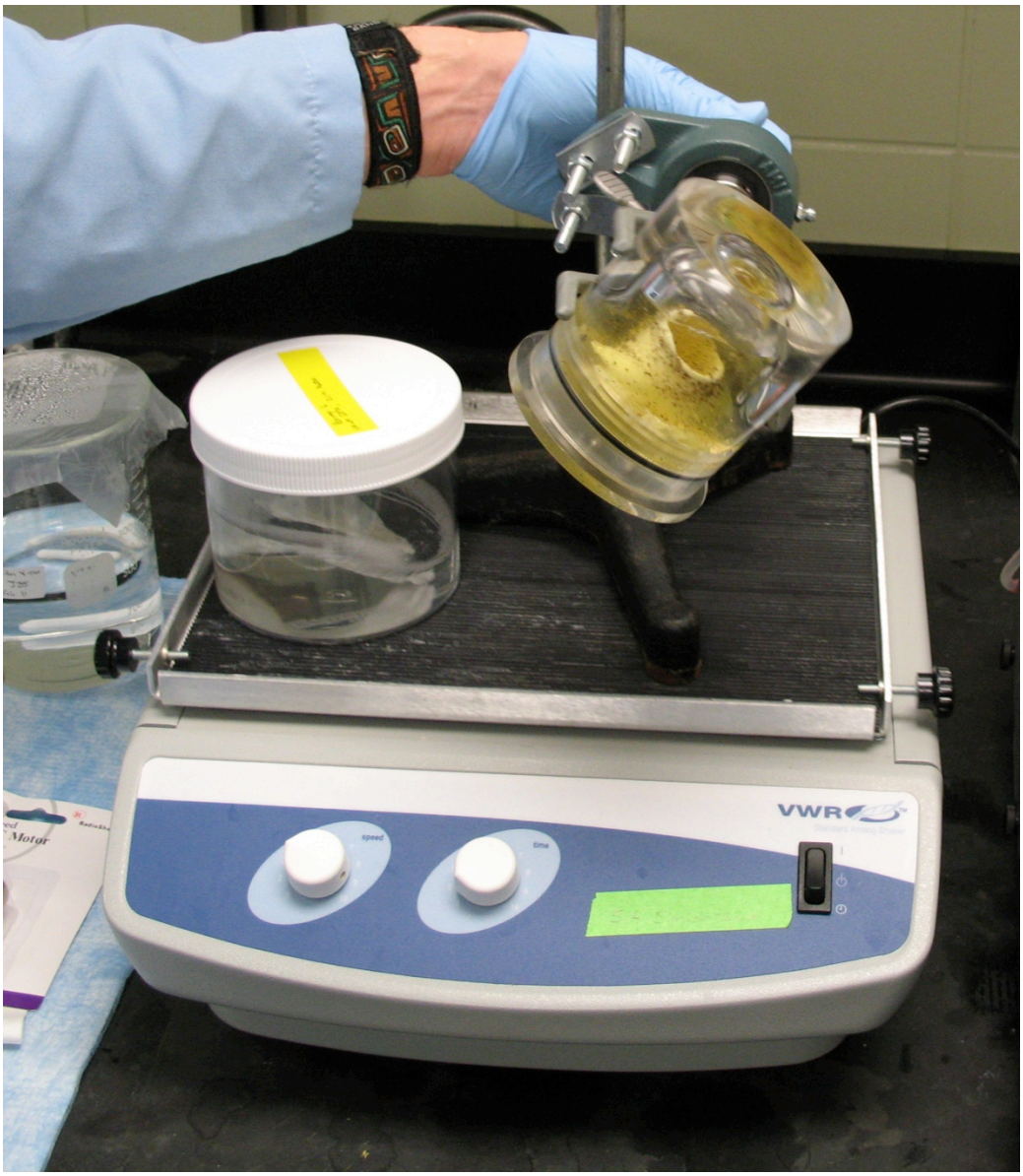


Figure 25: Study 1: Hand Rotation of Seeding Chamber

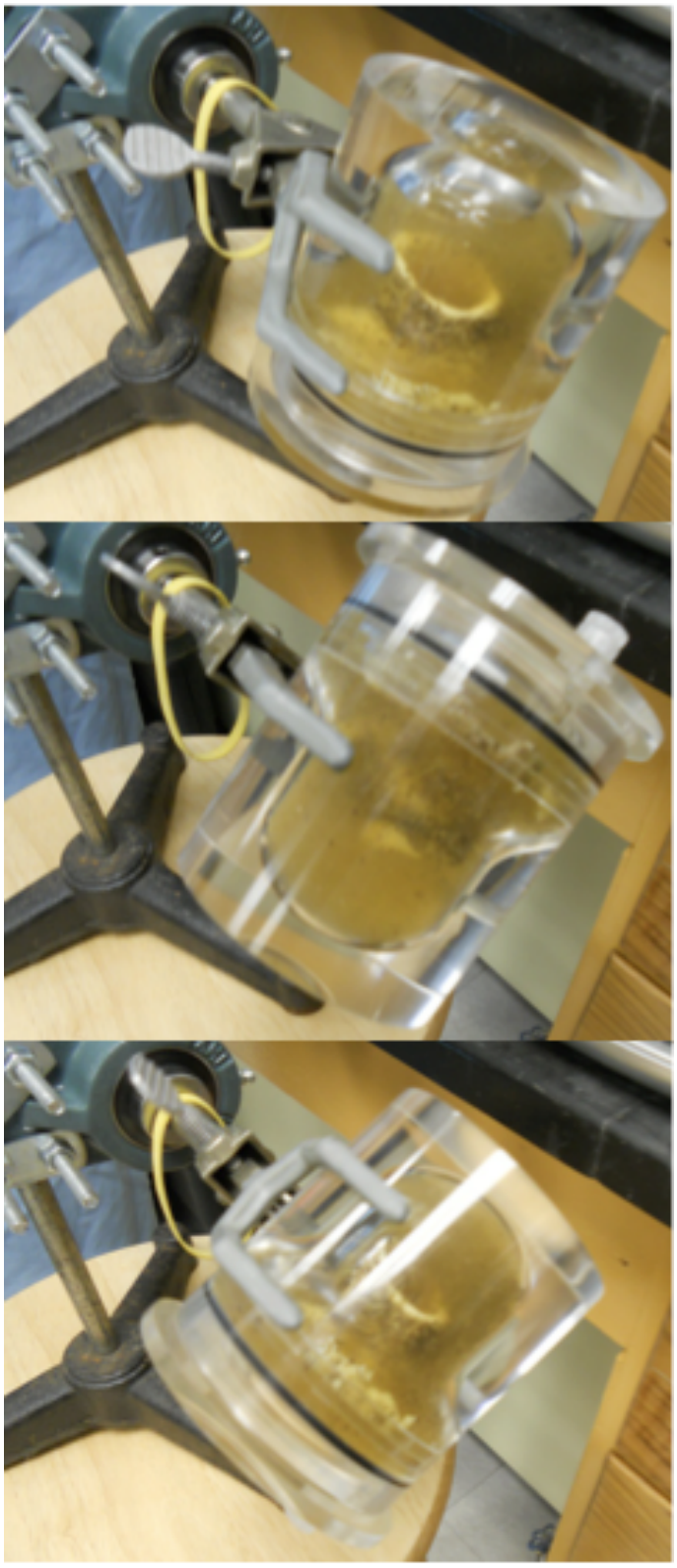


Figure 26: Study 1: Black Pepper Visually in Suspension during Rotation

3.4.2 Porcine Aortic Endothelial Cell Seeding for Studies 2-6

After expanding cells to desired numbers, the task of seeding cells onto the surface of the decellularized scaffolds was performed. Prior to seeding day, the dynamic seeding chamber was sterilized via a 12 hour ethylene oxide treatment. Mounting tools were sterilized through a one hour autoclave treatment. The decellularized, PGG stabilized scaffolds were pretreated in either a neutralization medium of 50% FBS and 50% MCDB 131+1% Ab/Am for Studies 2-5 or a 150μg/ml fibronectin solution overnight for Study 6. Under sterile conditions in a laminar flow hood, the pre-treated scaffold was mounted utilizing the mounting rings and place into the seeding chamber. The cell suspension solution was added to the seeding chamber in the incubator for the study specific dynamic protocol.

3.4.3 Study 2: Initial Low Concentration Cell-Seeding Study

Following Study 1 and general proof of concept, a second experiment was designed, which focused on a low cellular concentration seeding study. Under sterile the porcine aortic heart valve scaffold was mounted in the dynamic seeding device along with 1 million cells for a seeding density of 10,000 cells per milliliter (Figure 27). The seeding chamber was placed in the incubator on the shaker plate and rotated via an electric handheld screwdriver connected to a power supply allowing for variance of current to control the speed of rotation (Figure 28). The screwdriver and orbital shaker were to maintain a constant speed throughout the seeding time of 2 hours. Following this time point, the valve was removed from the seeding chamber and the cell seeding

suspension was spun down, re-suspended, and counted using the Scepter ™ Appendix B **(6.2.3).**

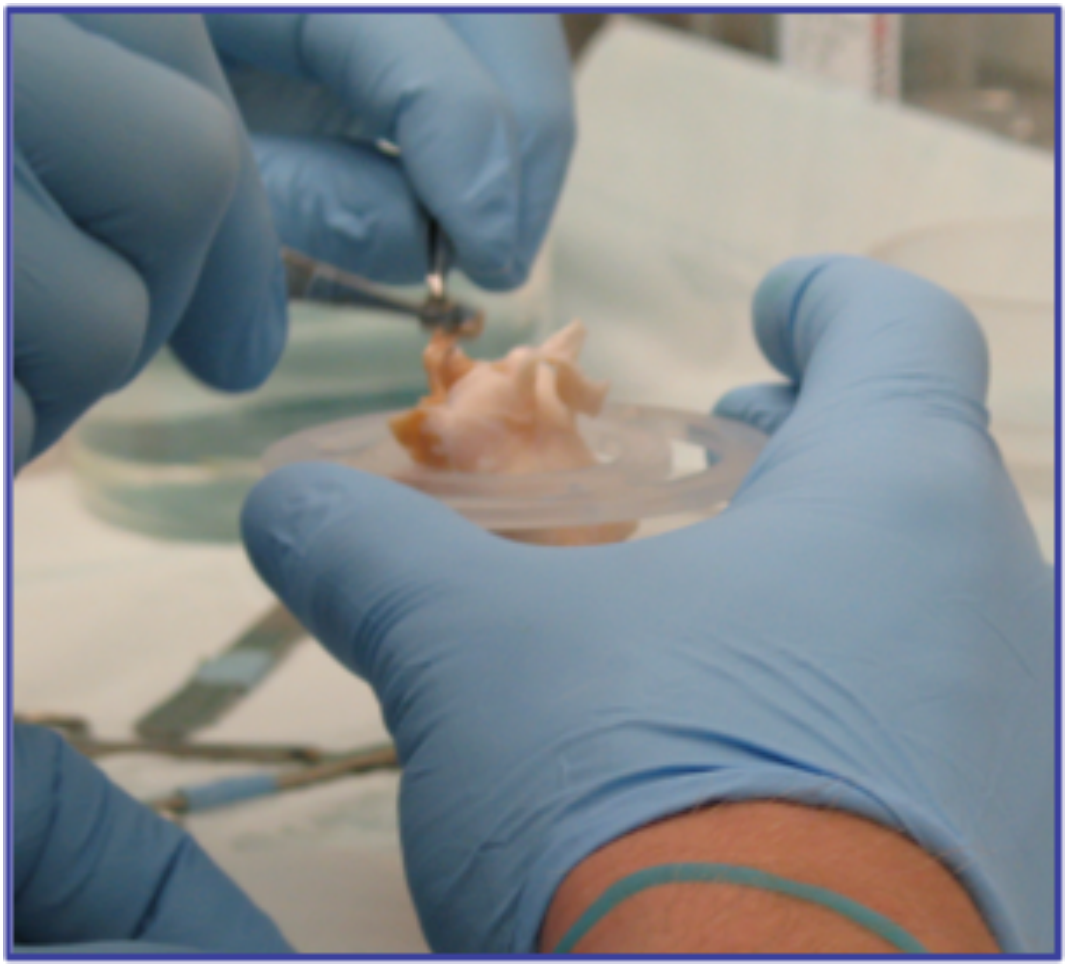


Figure 27: Mounting of Heart Valve Scaffold onto Mounting Ring

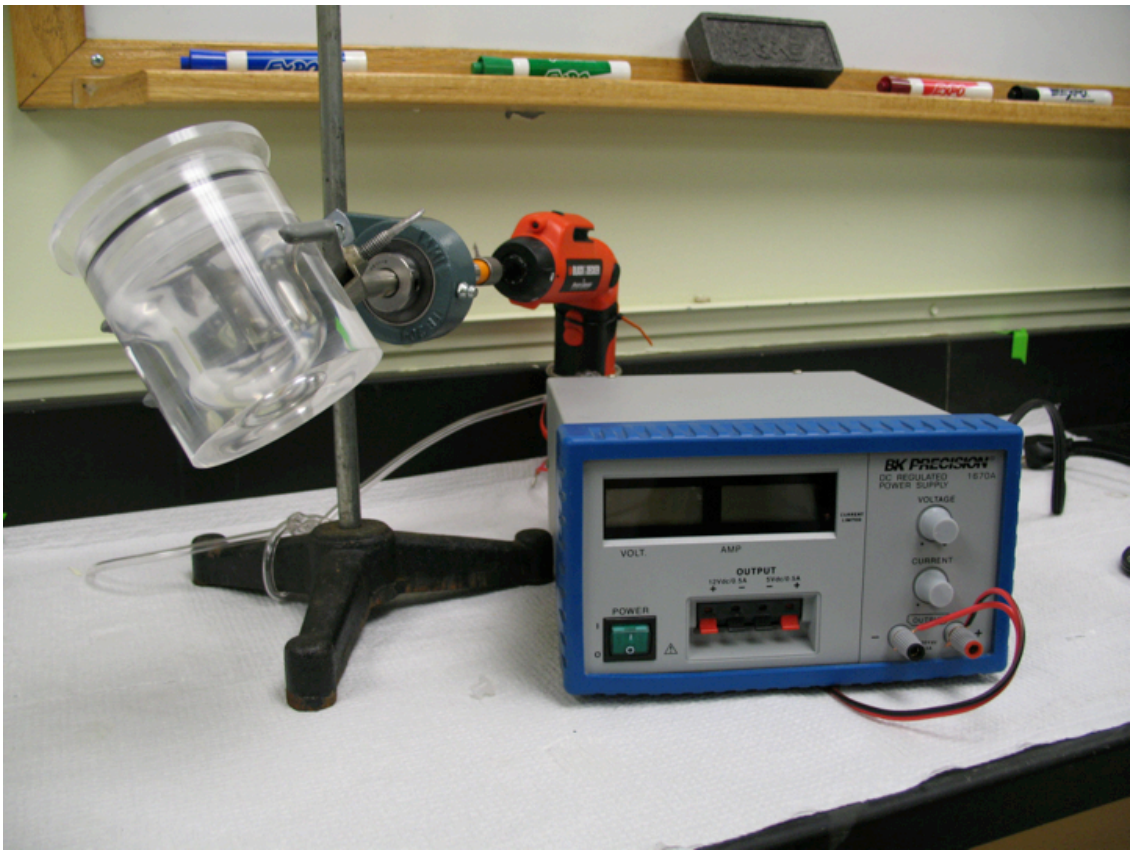


Figure 28: Study 2 Rotational Device Setup with Drill Motor and Power Supply

3.4.4 Live/Dead Assay

Following the removal of the seeded heart valve in Study 2 from the seeding chamber it was analyzed using The LIVE/DEAD Viability/Cytotoxicity Assay Kit (Life Technologies, Carlsbad, CA) which is a two-color fluorescence cell viability assay. Utilizing a fluorescence microscope and two fluorescence dyes live cells fluoresce green and dead cells fluoresce red. Live cells enzymatically convert non-fluorescent calcein AM into intensely fluorescent calcein giving off a green fluorescence. Dead cells fluoresce red as EthD-1 binds to nucleic acids of cells with damaged membranes. The

Live/Dead Assay was also used in analyzing the seeded scaffolds in subsequent Studies 3-6. See Appendix D **(6.4.1)**

3.4.5 Study 3: Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity

Study 3 was designed to address these issues of non-uniformity in the cell coverage as well as pattern of cellular attachment. Study 3 was performed in a similar manner to 2 with the following changes; cellular concentration was increased to 6.5 million or 65,000 cells per milliliter (Figure 29-Figure 31). The seeding chamber was rotated clockwise for 15 minutes followed by a 5-minute static period. The seeding chamber was then rotated 120 degrees and a counter clockwise 15 minute rotation followed by a 5 minute static period. Again the chamber was rotated 120 degrees and the direction was reversed. This process was repeated until 2 hours was reached. The 120 degree rotation was implemented to ensure each cusp spent an equal amount of time in each position about the rotational axis. The goal of this study was to see if a simple rotation, short 5-minute static period, and reversal of direction every 15 minutes could improve coverage.

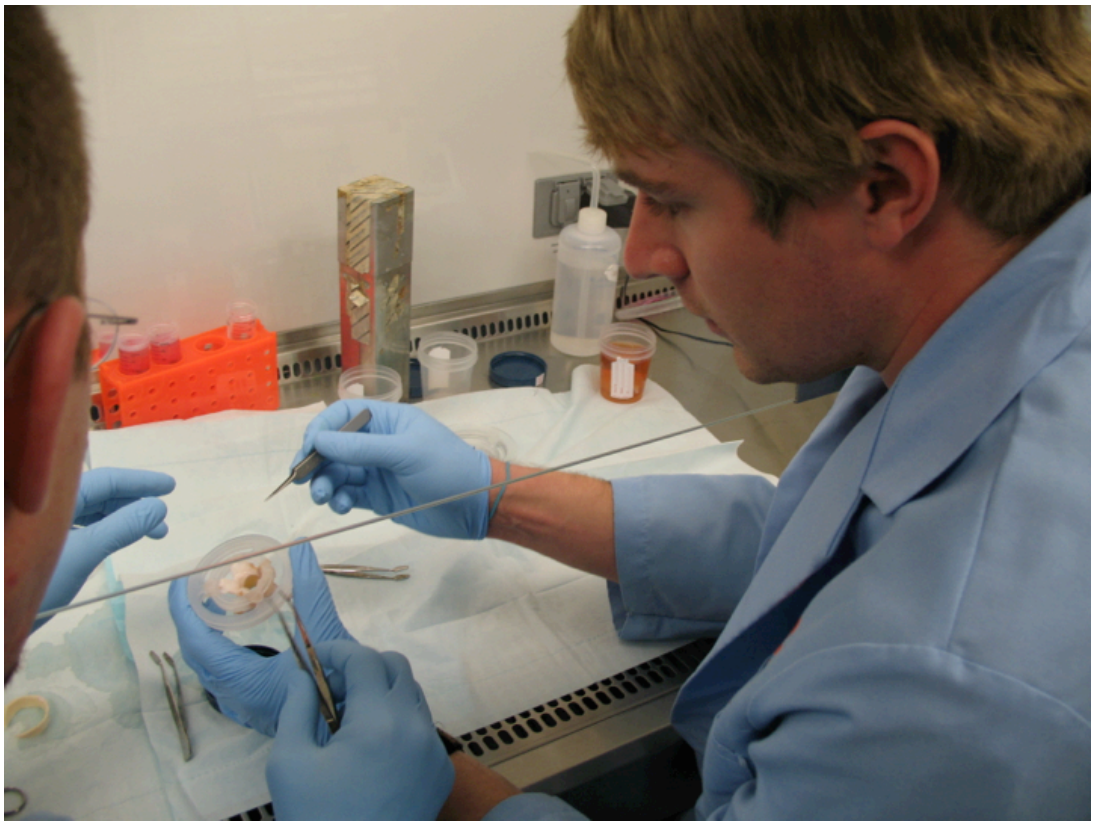


Figure 29: Sterile Mounting of Decellularized Heart Valve Scaffold into Cell-Seeding Mounting Rings

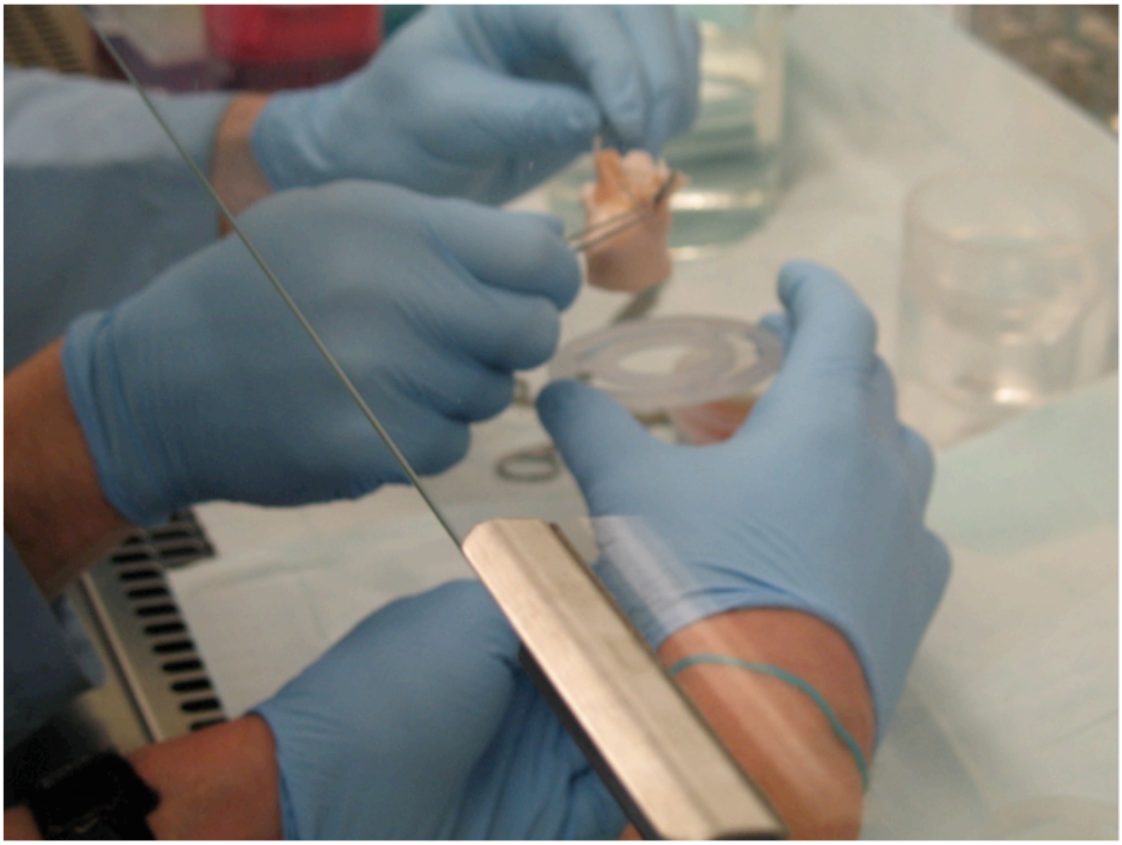


Figure 30: Mounting Heart Valve into Cell-Seeding Mounting Rings

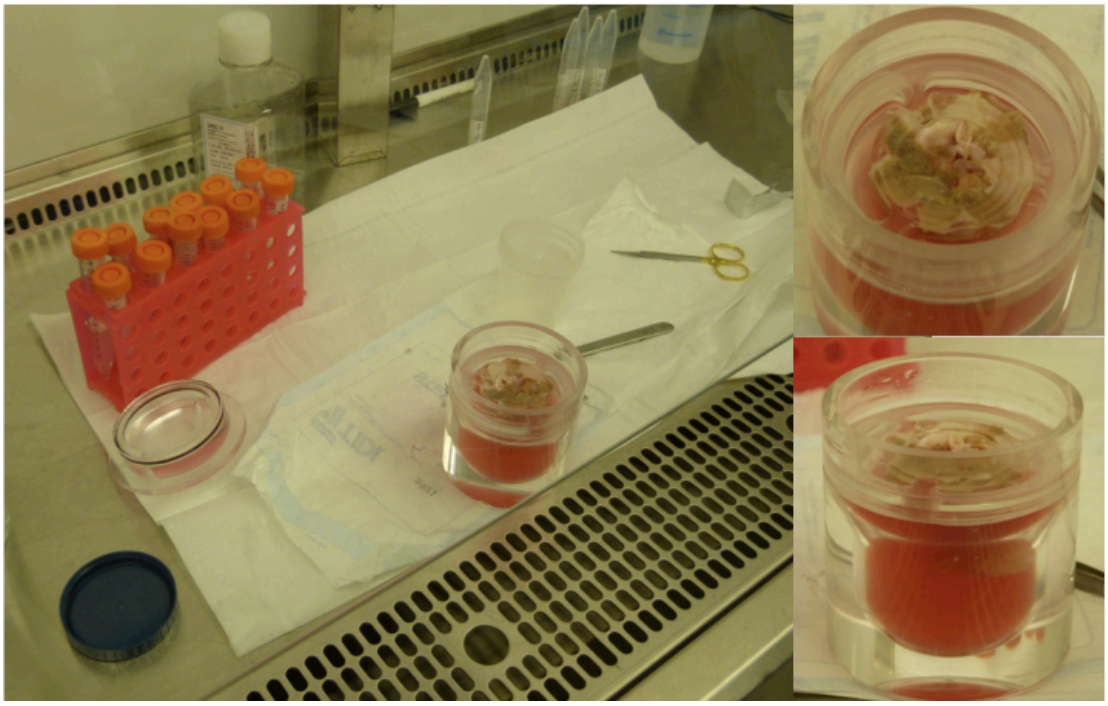


Figure 31: Study 3: Mounted Heart Valve in Dynamic Seeding Chamber

3.4.6 Study 4: Computer Control For Increased Coverage and Variable Seeding-Time Study

To this point, the results of the studies had yielded a working protocol for dynamic seeding using our chamber design. However there was still variability in the repeatability of trials that could be improved upon initial seeding chamber design.

Incorporating some changes into the design, the device was upgraded with the addition of an aluminum a-frame that attached to the orbital shaker plate through the use of factory shaker plate hardware. With this frame came the construction of two additional identical chambers resulting in the ability to seed three valves concurrently. The sample size had now increased to three while ensuring the valves experienced the exact same motion and environmental conditions. An electronic control unit was added

to control the rotational motion of the device (Figure 32). Through the use of a custom written LabView software program with a visual interface, a microcontroller, and a stepper motor, the rotational seeding device became much more uniform in its direction, speed, and duration of rotation (Figure 33).

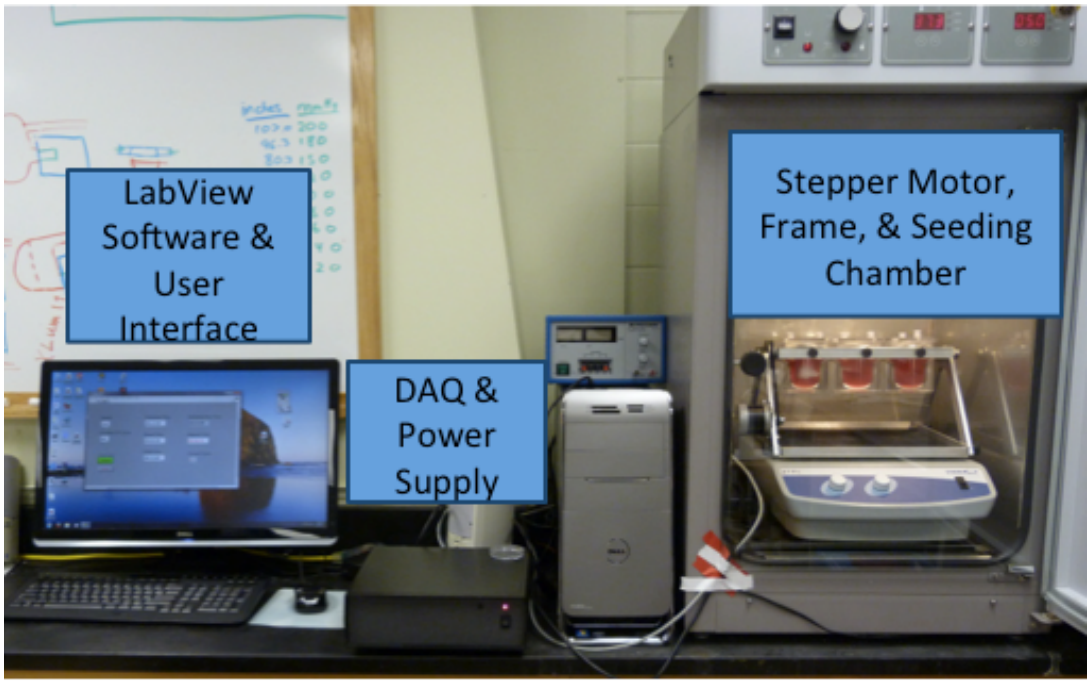


Figure 32: Upgraded Dynamic Seeding Device Overview

Figure 33: Dynamic Seeding Device Software Interface

Looking to improve upon the amount of unseeded cells remaining in seeding solution suspension led to the development of Study 4. In this study, the previous seeding times of 2 hours was to be investigated aiming to reduce the number of cells remaining in suspension by allowing for more cell scaffold contact. In addition, since there is no gas exchange into the chamber during seeding, there was concern about the possibility of hypoxia if the seeding time was too long. One decellularized PGG stabilized porcine aortic heart valve was placed in each of the three seeding chambers under sterile conditions. Each chamber was filled with 100 milliliters of cell culture medium along with 8 million cells. The seeding concentration for this run was held identical for each valve while the dynamic seeding time varied between the valves. All three valves began their dynamic rotation under identical conditions at the same time.

The LabView software was programed for a 30 minute clockwise rotation and 30 minute counterclockwise rotation with a 10 minute static incubation and 120 degree chamber rotation about the aortic axis between each change in direction.

After 2-hours, the first valve was removed from the frame and placed in a sterile sample cup in the incubator for a one hour static incubation before the cusps were cut from the valve. Live/Dead imaging was done on one cusp and a portion of the aorta while the remainder of the valve was placed into Karnovsky's fixative for Scanning Electron Microscopy (SEM) analysis. Following 4-hours of seeding, the second valve was removed from the frame and underwent the same treatment. After 6 hours the last valve was also removed undergoing the same treatment (Figure 34).

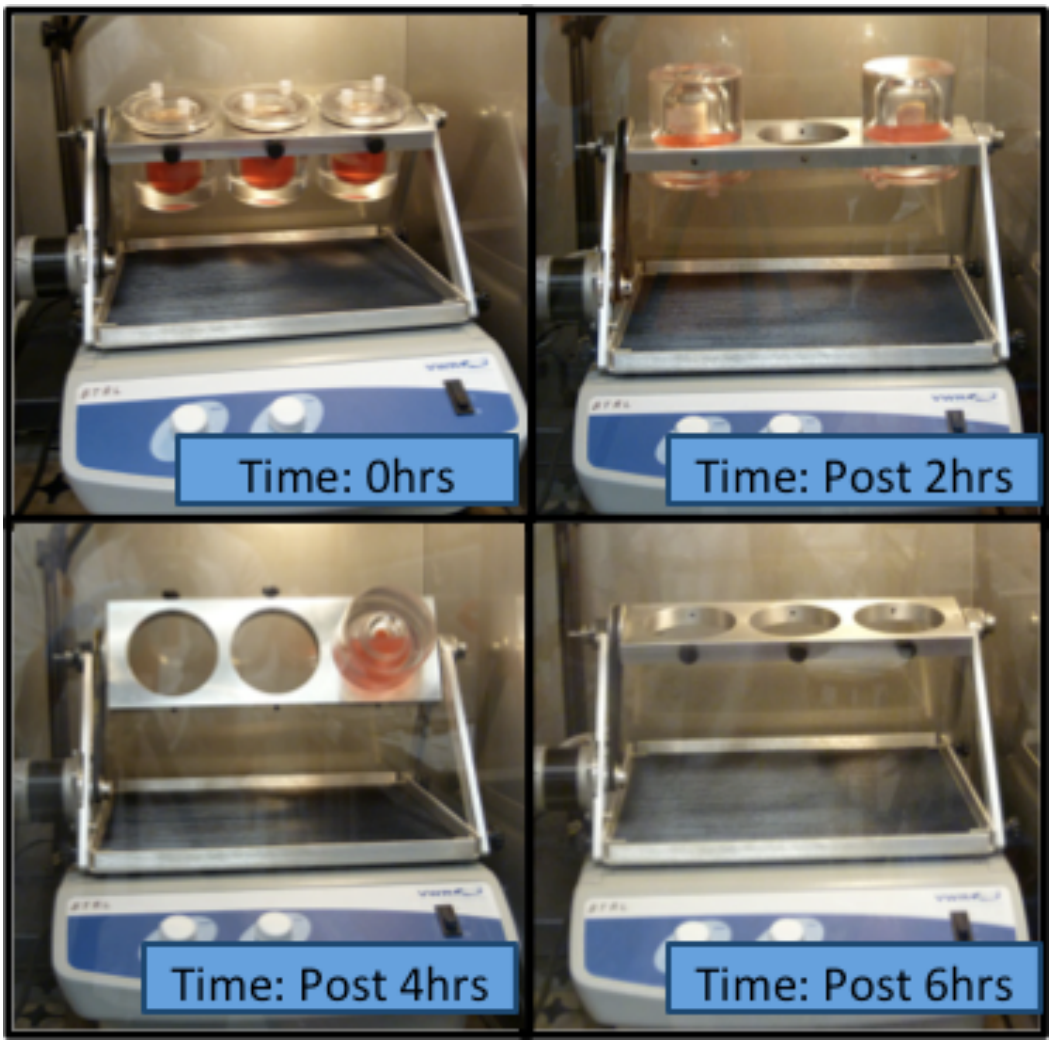


Figure 34: Study 4 Variable Seeding Time-Lapse

3.4.7 Scanning Electron Microscopy (SEM) Sample Preparation

Scanning electron microscopy was used to image the surface providing a high-resolution view of attached cells. Following the removal of the seeded scaffold in Study 4 from the seeding chamber, the cusp and portions of the aorta were carefully cut away from the valve and stored in Karnovsky's fixative (2.5% gluteraldehyde, 2% formaldehyde in 0.1 cacodylate, pH=7.4). Prior to SEM analysis, samples were dehydrated with ethanol. This was followed up by a critical point drying step using Hexamethyldisilazane (HMDS). Once fully dehydrated, samples were mounted, platinum sputter coated, (Figure 35) (Anatech USA 2012), and imaged using the Hitachi TM3000 (Figure 36) (Hitachi High-technologies 2010). The SEM procedure was also used to analyze sample from Studies 5 and 6. See Appendix D**: (6.4.2)**

Figure 35: Anatech USA Hummer 6.2 Sputter Coating (Anatech USA 2012)

Figure 36: Hitachi TM-3000 Scanning Electron Microscope (Hitachi High-technologies 2010)

3.4.8 Study 5: Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions

Following a successful seeding stage, and prior to achieving a functional implant, *in vitro* mechanical conditioning must take place to condition the cells for their native environment. Bridging the seeding to conditioning gap is a major step in the overall tissue-engineered heart valve paradigm. Previous and ongoing work BTRL by Lee Sierad has produced a pulsatile heart valve bioreactor (Figure 37) (Sierad et al. 2010). The heart valve bioreactor maintains viability of the implant by facilitating control over several parameters including dissolved oxygen and carbon dioxide, pH, temperature, nutrient concentrations, flow waveform, stroke volume, and physiological pressures found within the human heart. The heart valve bioreactor provides both a biochemical and mechanical conditioning environment necessary for this viability maintenance. The mixing movement of the medium is seen to provides deeper gas and nutrient exchange into the mounted scaffold (Lichtenberg et al. 2006; Gandaglia et al. 2010; Sierad et al. 2010).

The purpose of Study 5 was to see how the seeding step would transition into the next stage of dynamic conditioning. Focusing on cellular retention under shear stress and systemic pressures. Two valves were seeded with 10 million cells each following the same protocol as outlined in study 4 which included 30 minute clockwise/counterclockwise rotation with 10 minute static pause and 120 degree chamber rotation protocol for a total of 6 hours. Following the 6 hour dynamic seeding, the two valves underwent a 1 hour static incubation in the cell culture incubator. After which,

one valve was taken into the hood and under sterile conditions mounted in the **heart valve bioreactor** which was filled with medium.

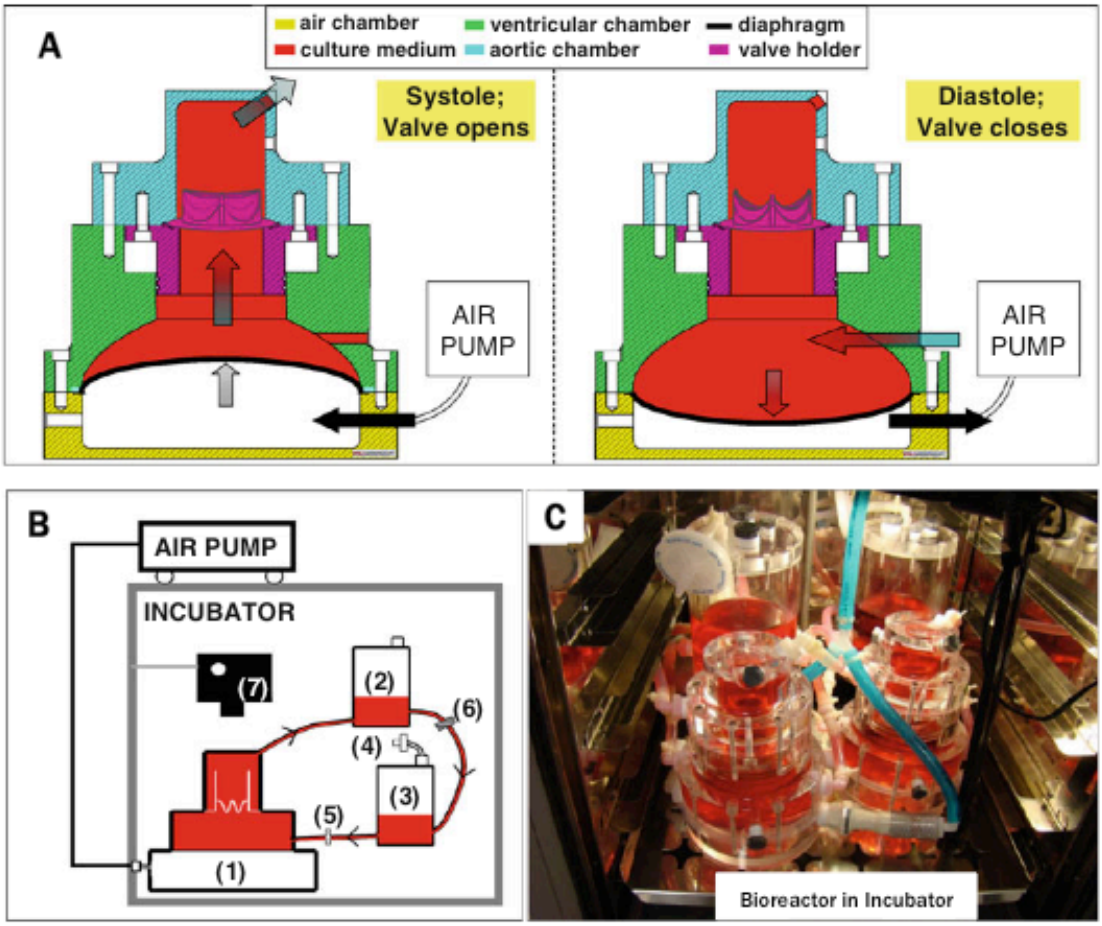


Figure 37: Heart Valve Bioreactor System. A) Medium Flow Through System B) Schematic Overview of Entire Bioreactor System: 1) Three-Chambered Heart Valve Bioreactor, 2) Pressurized Compliance Chamber, 3) Medium Reservoir Pump, 4) Sterile Filter for Gas Exchange, 5) One-way Valve, 6) Pressure Retaining Valve, 7) Webcam, and Ventilator Pump (Air Pump). C) Heart Valve Bioreactors with Mounted Cell-Seeded Heart Valves in the Cell Culture Incubator (Sierad et al. 2010)

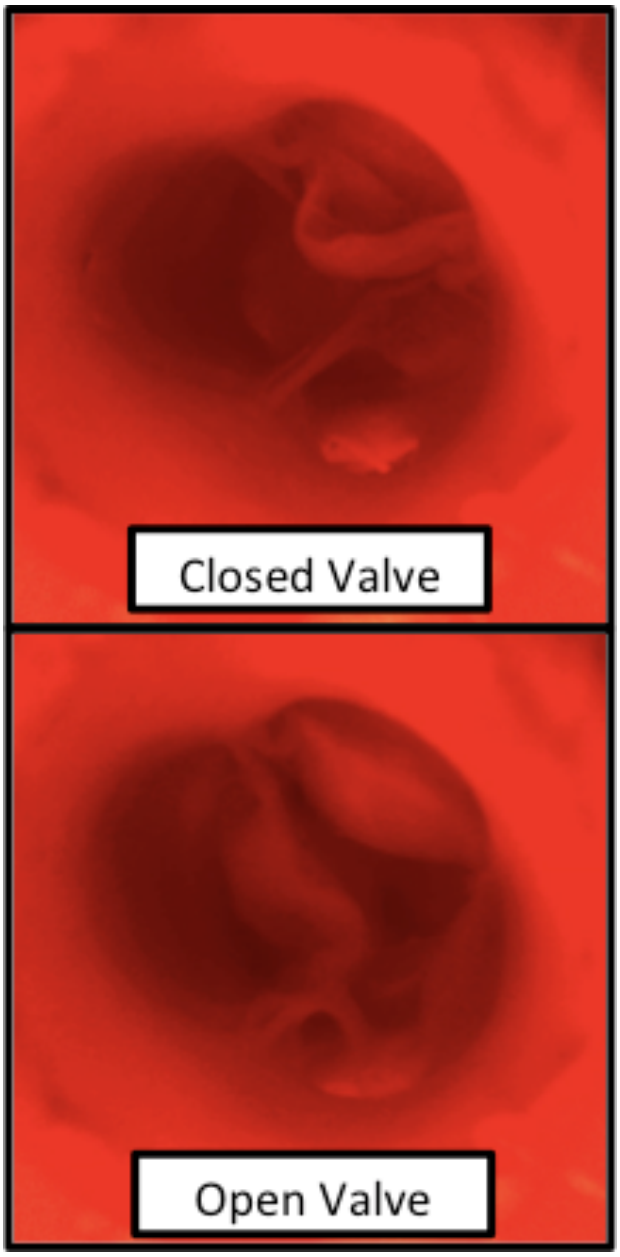


Figure 38: Cell-Seeded Heart Valve Mounted in the Heart Valve Bioreactor. Top Image: Closed Valve Under Diastolic Pressure. Bottom Image: Open Valve Under Systolic Pressure

The bioreactor was placed in the incubator and began to pump medium through the valve at 60 beats per minute and very low pressure which was ramped up to native aortic pressure of 120/80 millimeters of mercury according to Table 2 (Figure 38). The

valve was slated to run in the Bioreactor for 1 week and the medium was changed every 3 days.

The other valve was removed from the static 1 hour incubation and analyzed using Live/Dead Assay and SEM. This valve provided a post-seeding sample for analysis and comparison with the 1 week dynamically conditioned sample. Following 1 week the dynamically conditioned valve was removed from the bioreactor and analyzed using Live/Dead and SEM.

3.4.9 Study 6: Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions

Study 6 was set up similar to Study 5, with a couple of big changes, i.e., the addition of a fibronectin overnight treatment to the scaffold as well as an overnight static incubation replacing the previous 1 hour static incubation. Two scaffolds were submerged in a 2-microgram fibronectin per milliliter PBS solution and mounted to a rotisserie overnight. Following scaffold treatment, the seeding protocol described in Study 5 was run seeding 2.5 million cells per valve.

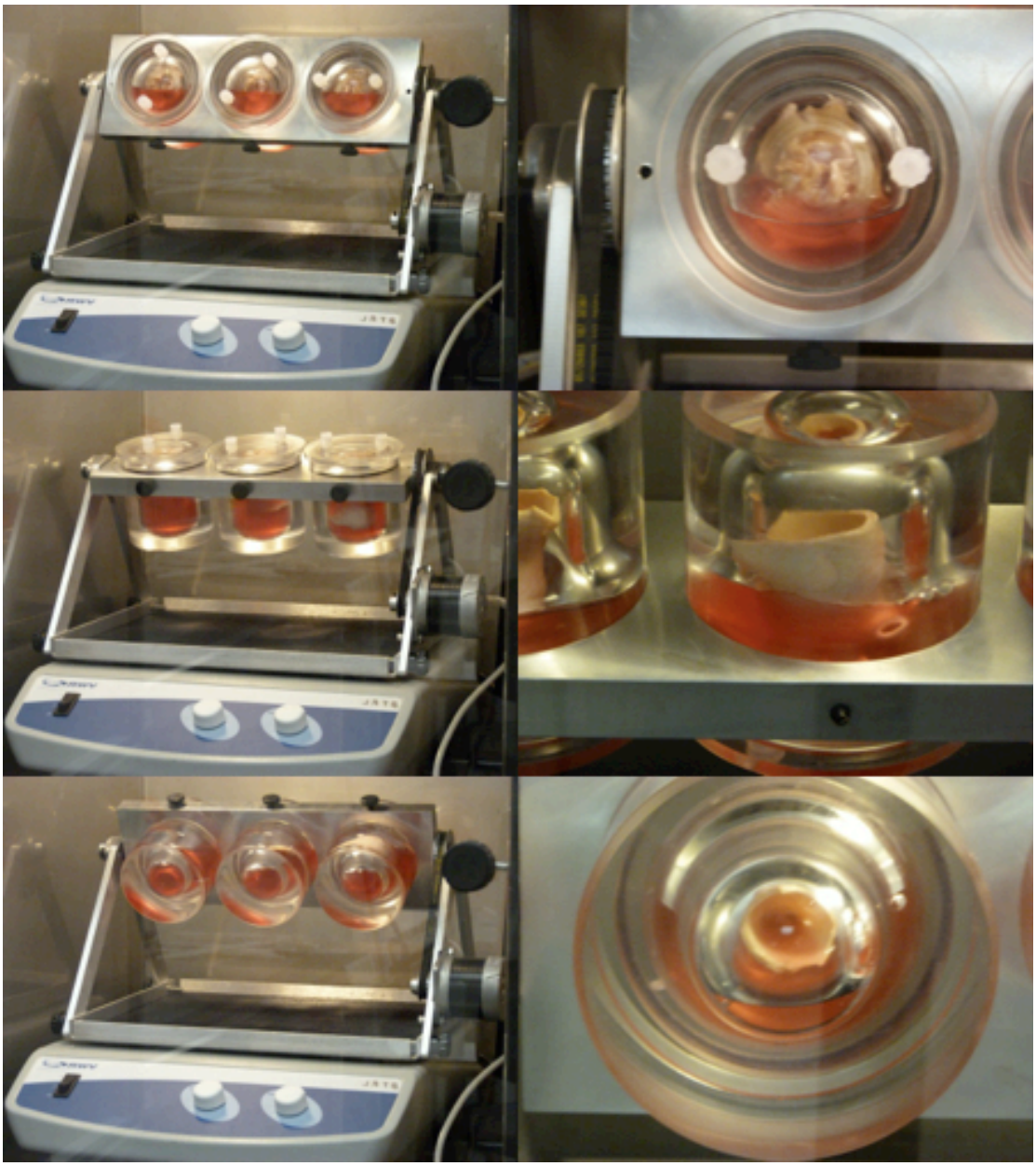


Figure 39: Macroscopic View of Mounted Heart Valves Under Rotation

Following the 6 hour dynamic seeding, the valves were placed in the incubator for a static overnight incubation (Figure 39). The following day, 1 valve was mounted in the bioreactor under sterile conditions and placed in the incubator. The pressure was set low and slowly ramped up according to Table 3.

Based on the results from Study 5, and noting cellular loss attributed to shear force and high systemic pressure values, the maximum pressure for Study 6 were limited to pulmonary pressures of 40/25 millimeter of mercury. The valve remained in the bioreactor for 2 weeks and the bioreactor medium was changed every 3 days. The second valve was removed and analyzed with Live/Dead Assay and SEM after the overnight static incubation. Following the 2 week time period, the conditioned valve was removed and also analyzed through Live/Dead Assay and SEM.

CHAPTER THREE

EXPERIMENTAL RESULTS AND DISCUSSION

4.1 Study 1: Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study)

The results from this experiment showed that a particulate substance similar to cells could be maintained in suspension with the type of dynamic motion of the seeding device. In addition to confirming the ability of maintaining a particulate such as cells in suspension, the optimal fluid volume was visually studied (Figure 40). Through varying the mock cell suspension levels from completely full down to a level where at times of rotation the valve was out of the solution and the medium failed to flow through the interior of the valve. From this, an ideal level was observed to give visually optimal flow through and around the valve. After determining the desired seeding suspension level, the mock suspension volume was measured to be about 100 milliliters. These results provided a proof of concept that our device was indeed capable of achieving a random non-violent flow of cell seeding suspension through the valve with the particulate contacting all surfaces of the valve. Also investigated in this study was the axis of rotation upon which the fluid would flow through and around the valve in an optimal controlled manor. By varying the position of the axis of rotation the best fluid flow was found to be centered on the local of the mounting ring through the plane of the cusps. This location worked best with the specified 100mililiter volume and the curves of the chamber to produce the most non-violent flow through and around the valve while contacting all surfaces.

This first study gave an important sense of where to set some basic parameters for future studies. Ideal fluid volume, axis of rotation, and a ballpark sense of rotational speed were all garnered.

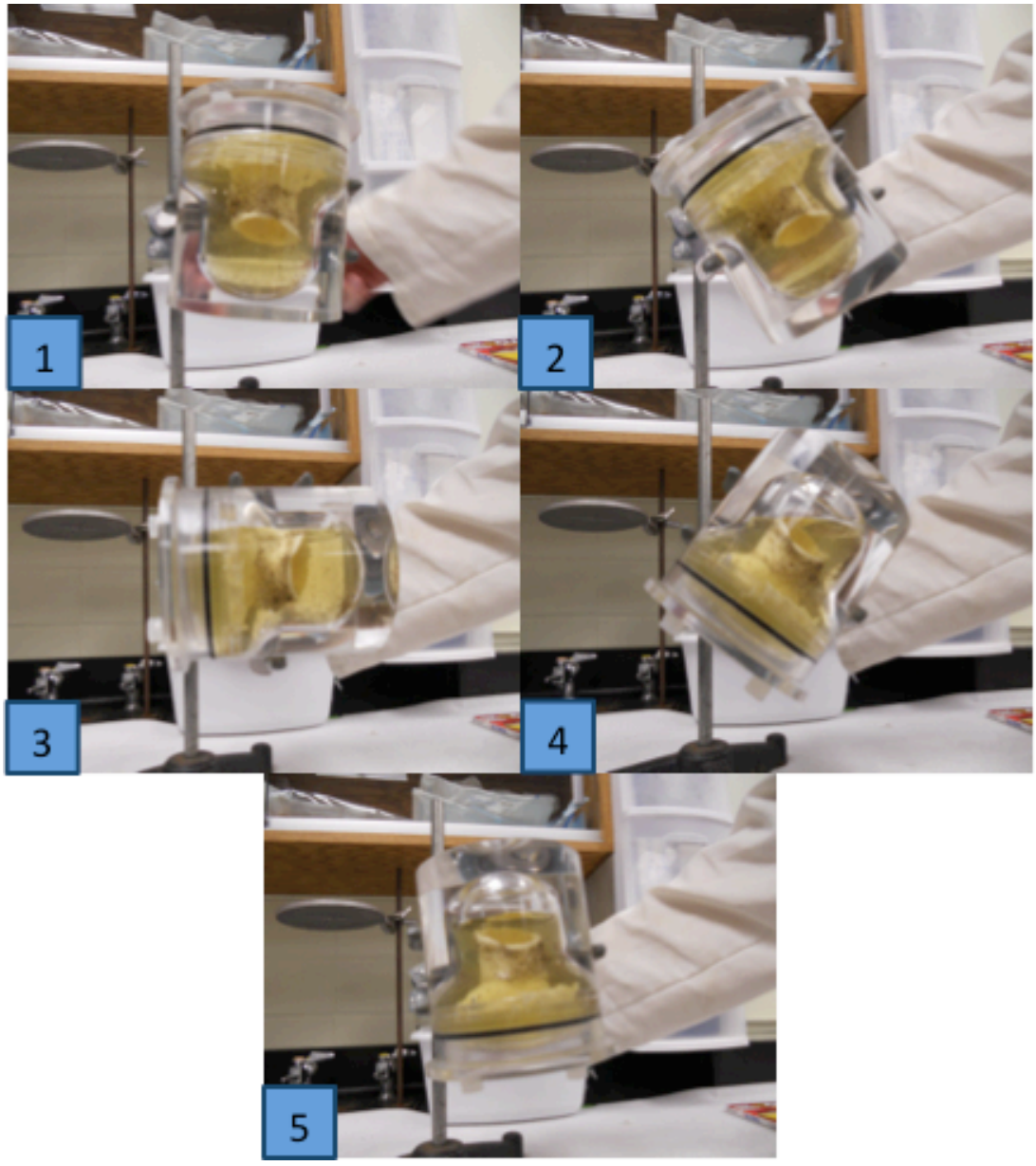


Figure 40: Study 1 Black Pepper Sequential Rotation Images

4.2 Study 2: Initial Low Concentration Cell-Seeding Study

It was found that of the 1 million cells seeded 180,000 or 18% remained in the seeding solution. Live/Dead assay was performed to observe the attachment and viability of cells on the surface. All three cusps were analyzed, looking for uniformity of coverage on both the ventricularis and fibrosa surfaces of the cusps as well as the lumen and adventitia of the aorta, and lastly the coronary and mitral surfaces. From the Live/Dead imaging a pattern was noticed where the fibrosa surface of one cusp had significantly more cells than its ventricularis counterpart (Figure 41). Similarly, the ventricularis surface of another cusp had far more cells than the cusps fibrosa surface. Based on this uneven coverage further improvement was required to increase uniform coverage. Looking back at the experimental set up, the cause of non-uniform cellular attachment and obvious pattern was hypothesized to be due to the unidirectional rotation and position of each cusp about the axis of rotation. As the cells fell out of suspension onto the valve surface, only one side was exposed to the settling cells. The variances in coverage were found to be affecting only the two non-axial positioned cusps (Figure 41: cusps 2 & 3), which further supported the hypothesis that the direction of rotation was at play. The opposing surface of adherence noted between the two cusps (Figure 41: cusps 2 & 3) indicated that the location of the cusps within the chamber relative to the axis as well as the unidirectional rotation must be taken into account in order to increase uniformity of cellular coverage.

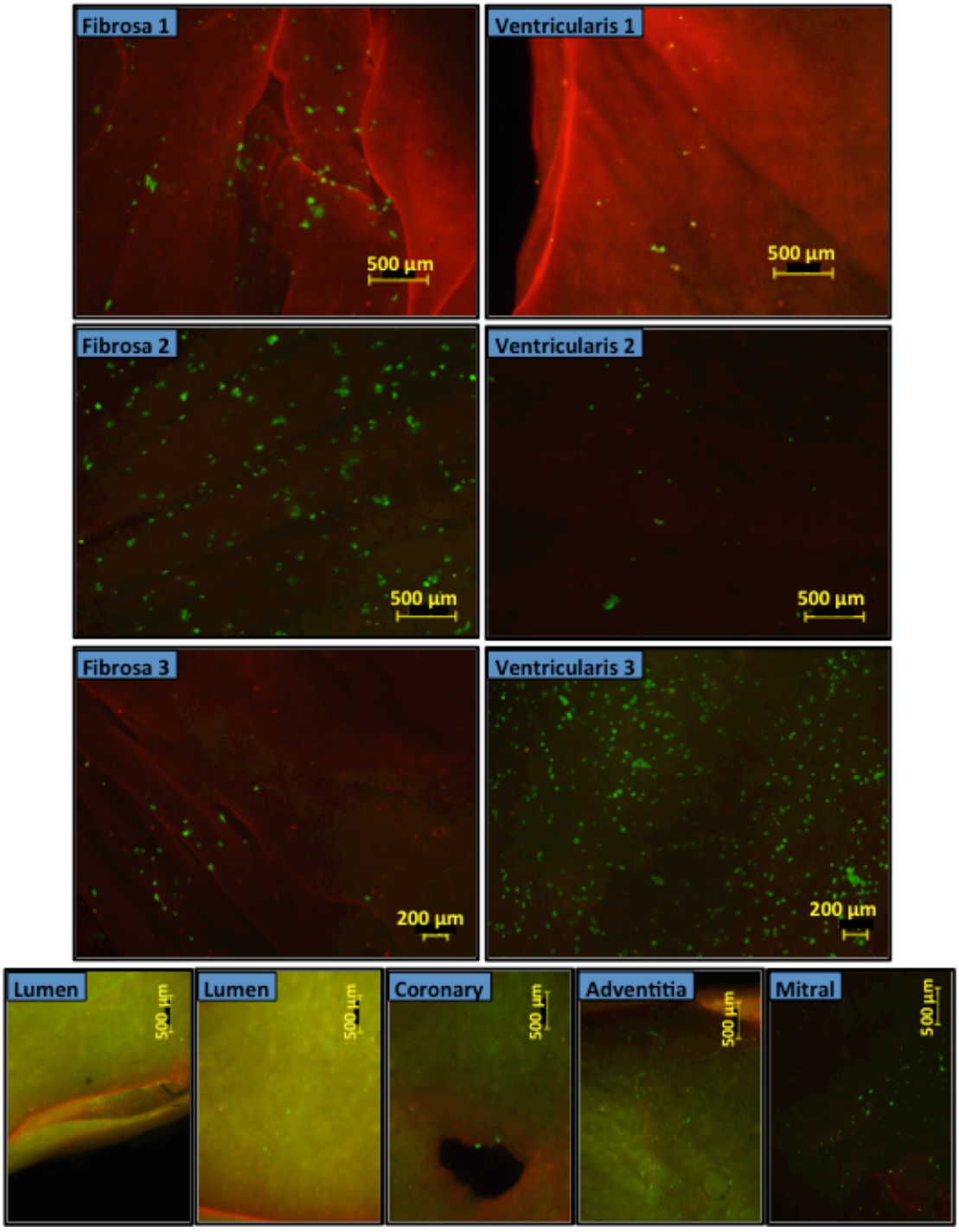


Figure 41: Study2: Live/Dead Assay; Axial Cusp=Fibrosa and Ventricularis 1. Non-Axial Cusps=Fibrosa and Ventricularis 2 & 3. Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

4.3 Study 3: Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity

The valve was removed and the cells suspension was spun down, re-suspended, and counted using the Scepter™; there were 2.5 million or about 38% of the original 6.5 million remaining in the seeding suspension. Live/Dead Assay was performed and the results compared to the patterning found in Study 2. The Live/Dead results indicated a more uniform cellular attachment on all 3 leaflets, fibrosa and ventricularis, as well as aorta and coronary sinuses (Figure 42).

The increase in percentage of unseeded cells may have been due to the much larger concentration of cells in solution and a crowding effect where many of the cells didn't contact the surface or have time to adhere. With 38% of the initial cells not seeded on the scaffold and still in suspension additional changes to the protocol with hopes of decreasing the number of unseeded cells were investigated. Study 4 was to address this key issue through the focus on increasing the seeding time.

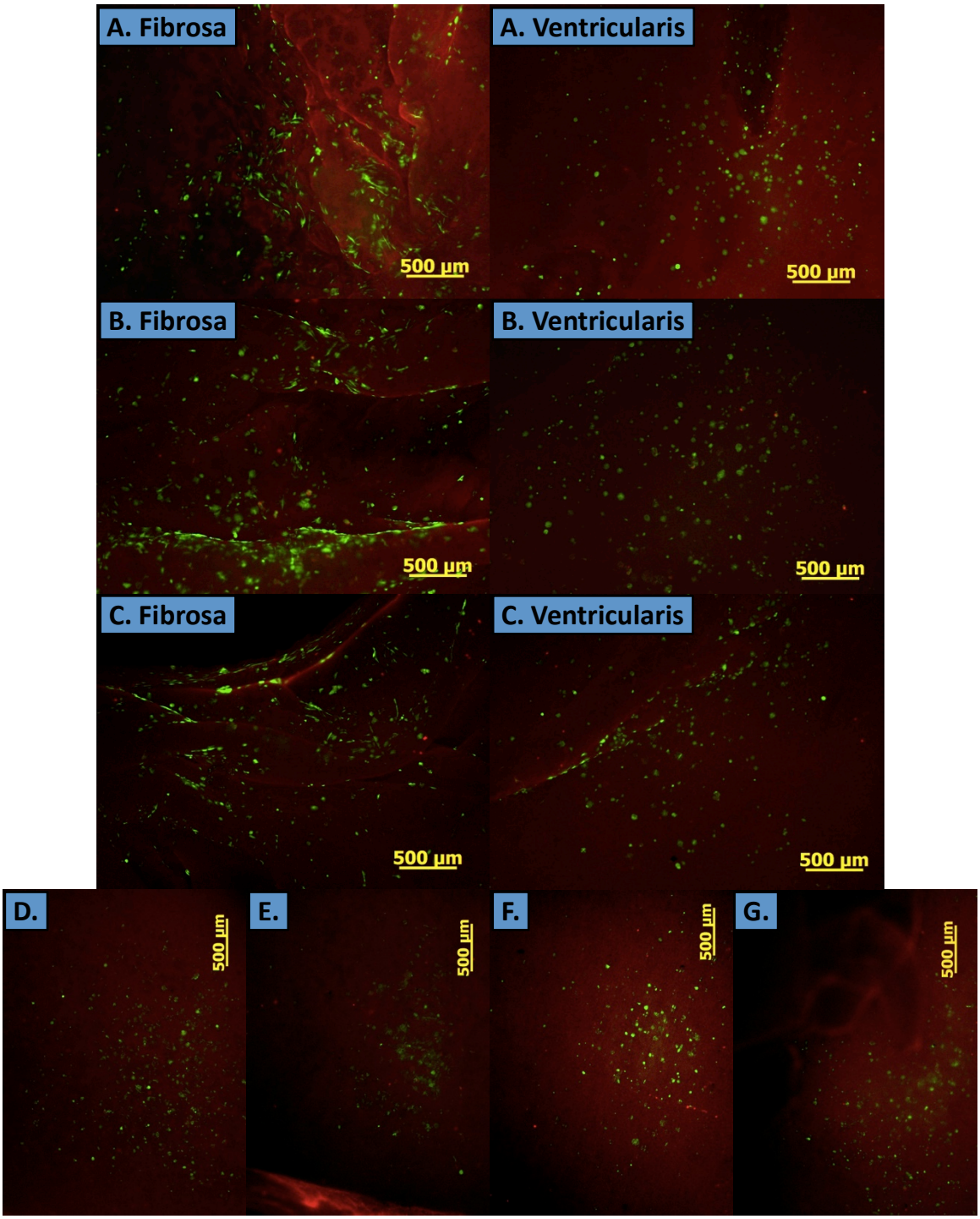


Figure 42: Study 3: Live/Dead Assay; Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red. A. Non-Coronary Cusp B. Coronary Cusp (Radial) C. Coronary Cusp (Axial) D. Coronary Artery (Axial) E. Coronary Sinus (Axial) F. Aortic Lumen G.Aortia, Cusp Junction.

4.4 Study 4: Computer Control For Increased Coverage and Variable Seeding-Time Study

Comparing the Live/Dead images from each of the 3 time points, it was determined that the 6-hour time point yielded better cell coverage and despite the longer seeding time there were no visible increase in cytotoxicity (Figure 43). SEM images were taken at a later date on the 4 hour (Figure 44) and 6 hour (Figure 45) samples and their results confirmed the initial observation that 6 hour time point yielded better coverage. Looking at the morphology of cells on the scaffold surface in the SEM images (Figure 44 & Figure 45), the 6 hour samples many cells appeared as if they were more spread on the surface compared to the rounded nature seen more commonly in the 4 hour samples. This is an ideal response and a good indication that the cells like the structure and composition of the decellularized PGG-stabilized porcine heart valve scaffold.

Following Study 4, the seeding device and protocol had shown its feasibility to dynamically seed cells on all surfaces of the valve. Looking toward future studies, the overall picture of an autologous tissue engineered heart valve and more specifically the stage of mechanical conditioning was chosen to be the goal of Study 5.

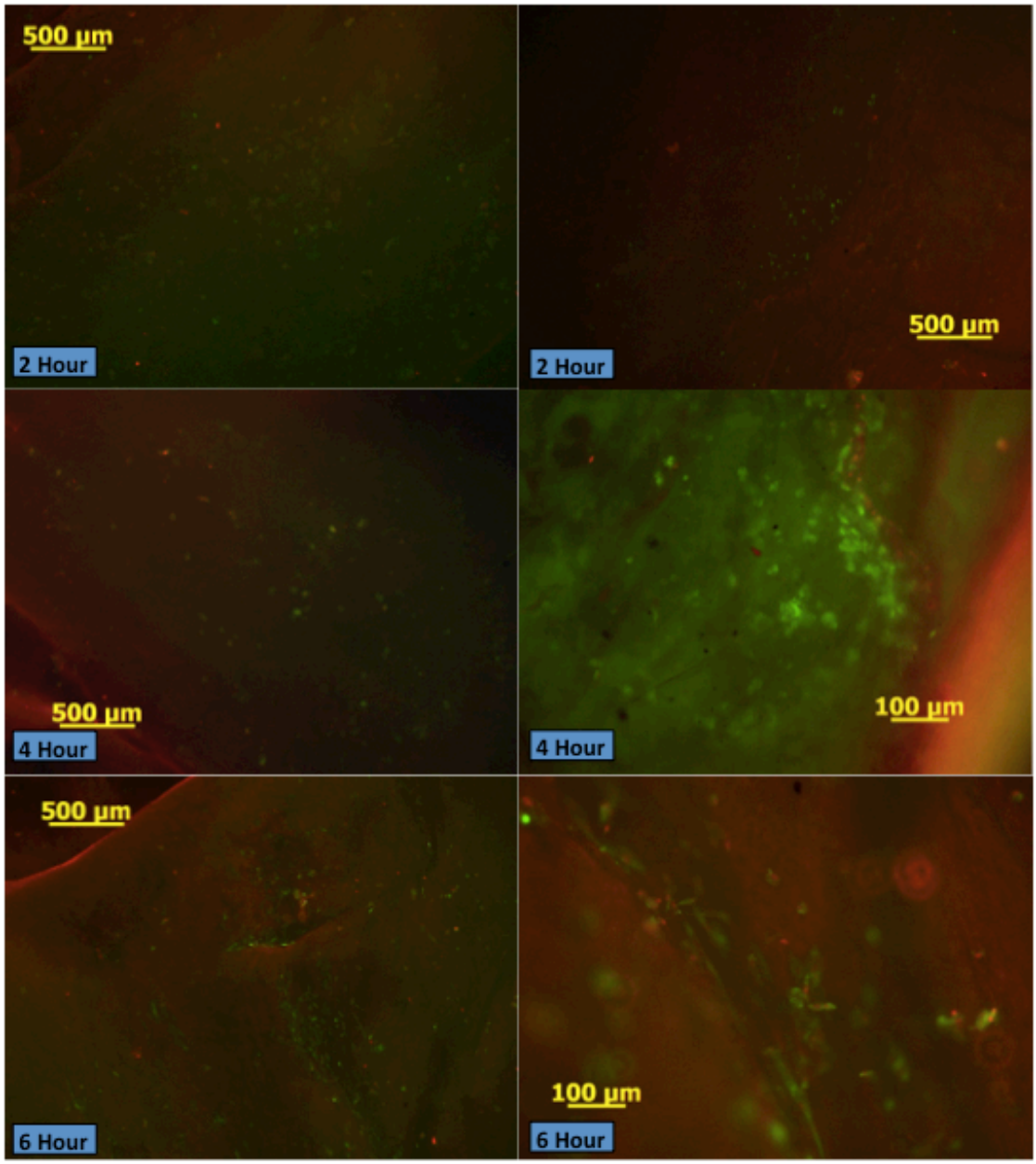


Figure 43: Study 4 Live/Dead Assay; Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

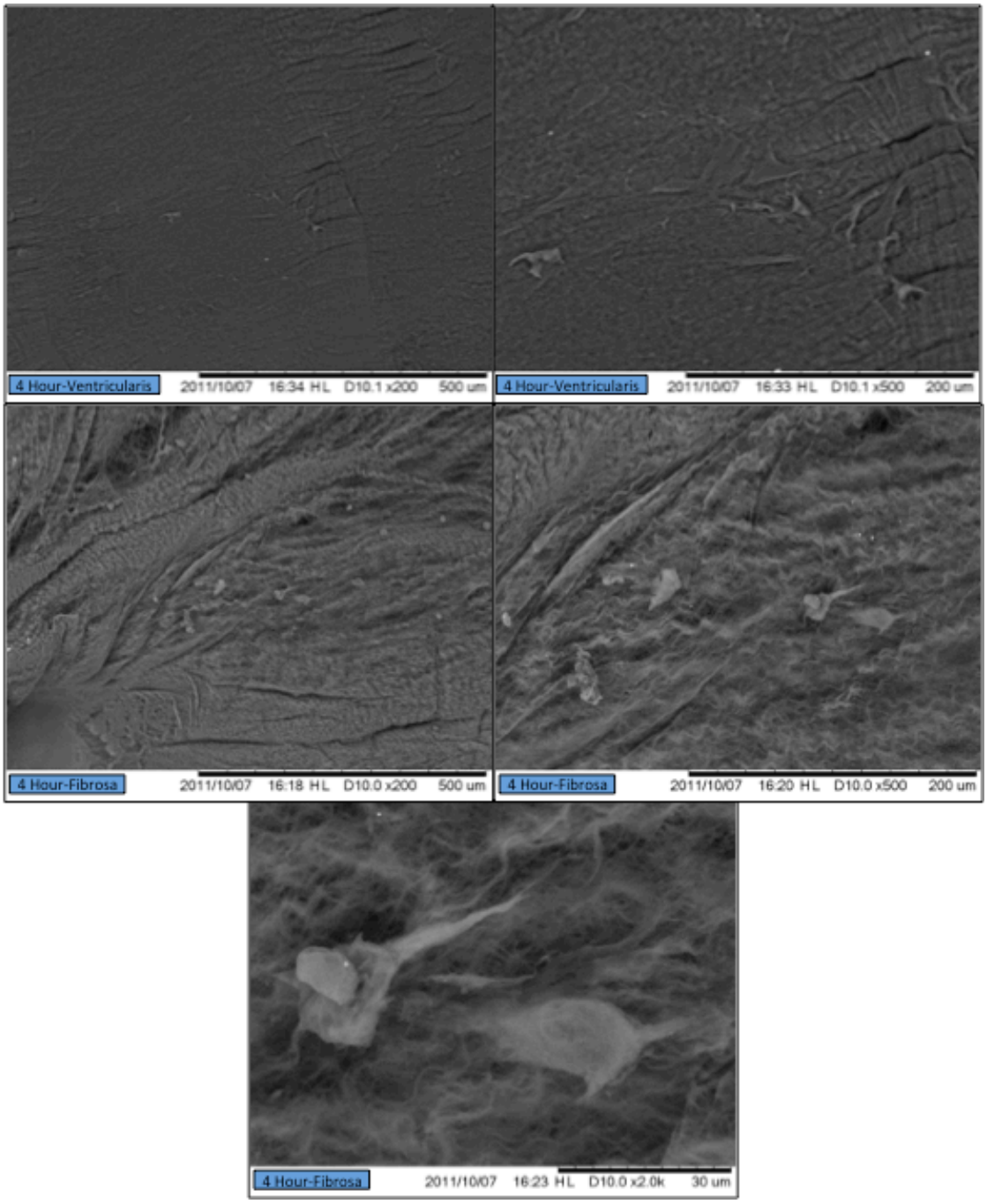


Figure 44: Study 4: 4Hour Sample SEM

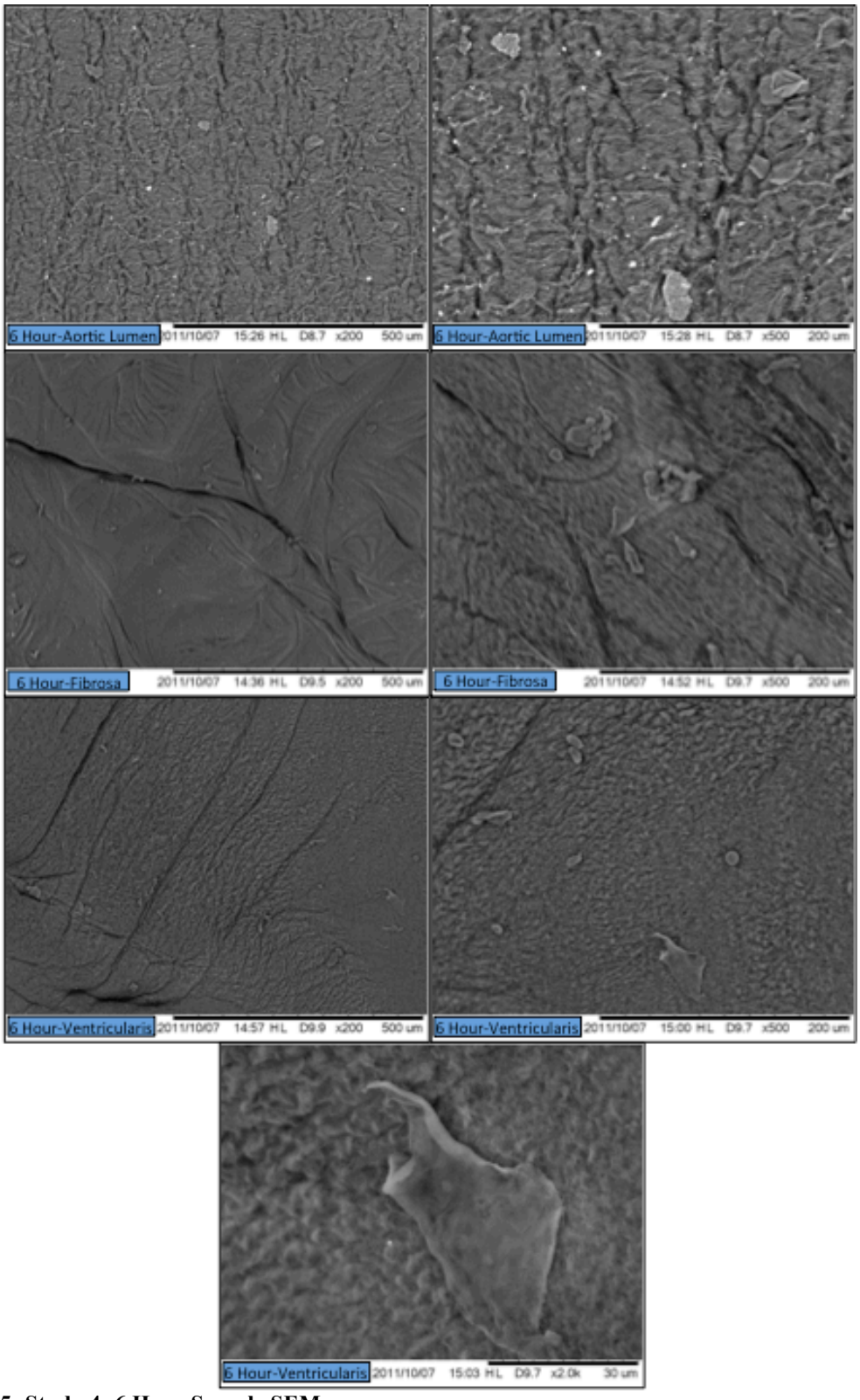


Figure 45: Study 4: 6 Hour Sample SEM

4.5 Study 5: Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions

Both the Live/Dead (Figure 46-Figure 51) and SEM (Figure 52-Figure 55) results showed many cells on all surfaces of the scaffold post 6 hour seeding as was expected based on the previous studies. Analysis of the 1 week dynamically conditioned valve yielded less than ideal results. Both Live/Dead and SEM showed very few cells were retained on the valve surface (Figure 56-Figure 61).

Results drawn from Study 5 were that many cells on the surface of the valve post seeding but may have been washed from the surface during bioreactor conditioning. These results lead to the focus of Study 6, and attempts to improve cellular retention on the surface through the conditioning stage.

Table 2: Pressure Values Over 1-week Conditioning

Date	Time	Pressure (mmHg)
12-Oct	23:00	10/7.5
13-Oct	11:30	15/7.5
	12:30	21/10
	16:30	31/15
	17:30	39/22
14-Oct	11:00	50/28
	14:40	60/40
	16:45	76/49
	21:10	90/62
	0:50:00	100/70
15-Oct	18:50	120/80
	18:55	118/83
	20:30	120/80
16-Oct	19:45	120/80

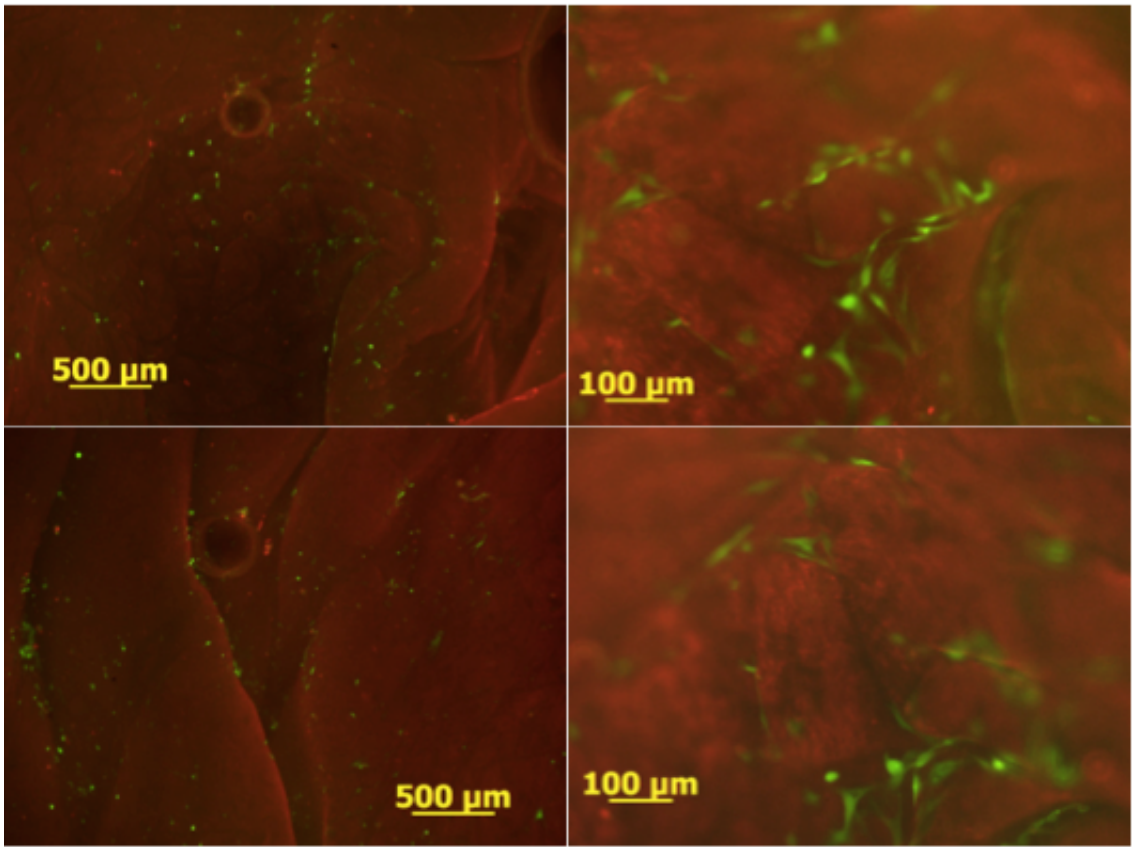


Figure 46: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation Live/Dead (Coronary Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

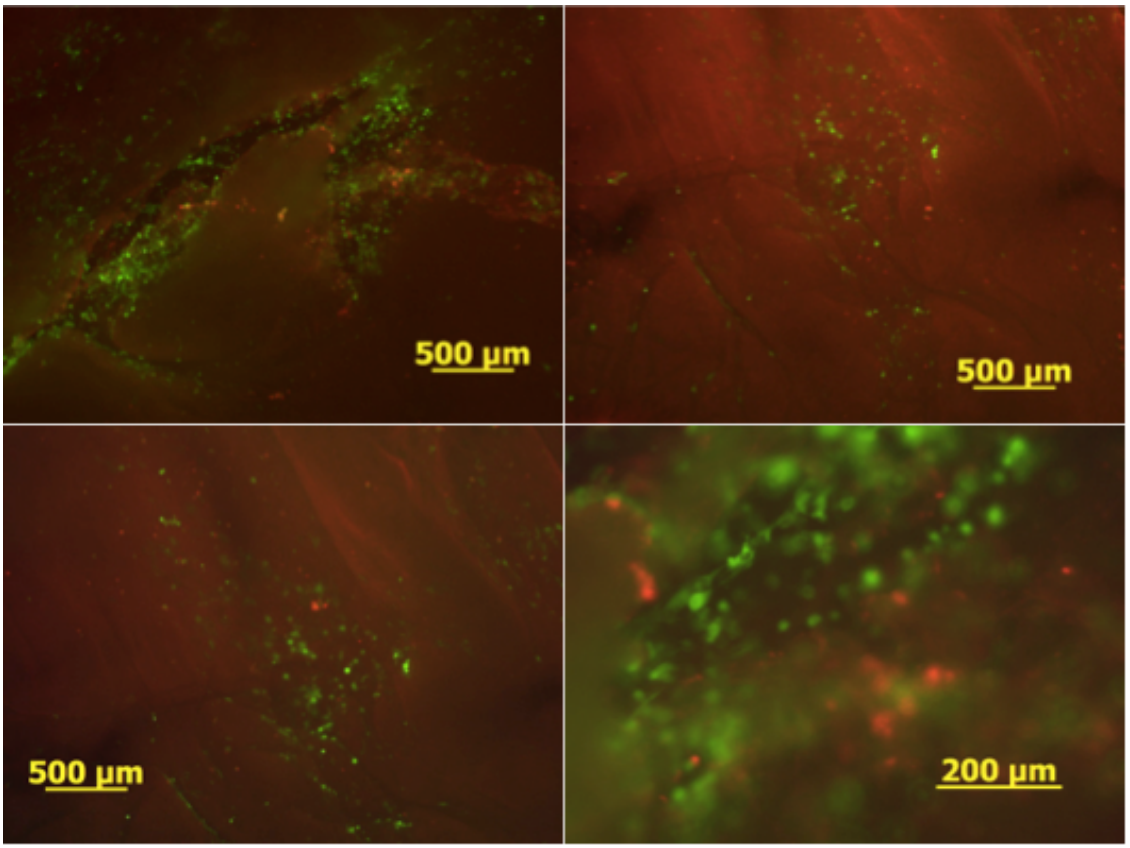


Figure 47: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation Live/Dead (Coronary Ventricularis). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

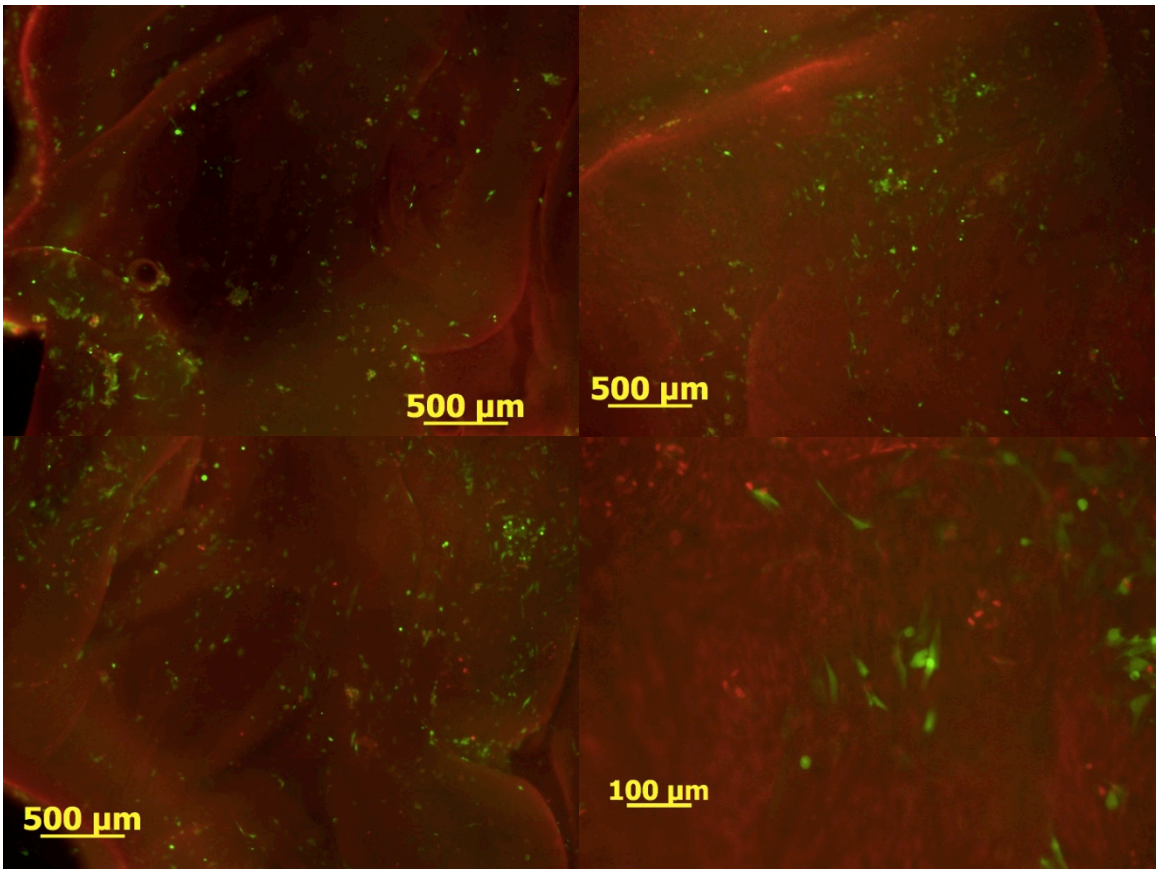


Figure 48: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation Live/Dead (Coronary 2 Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

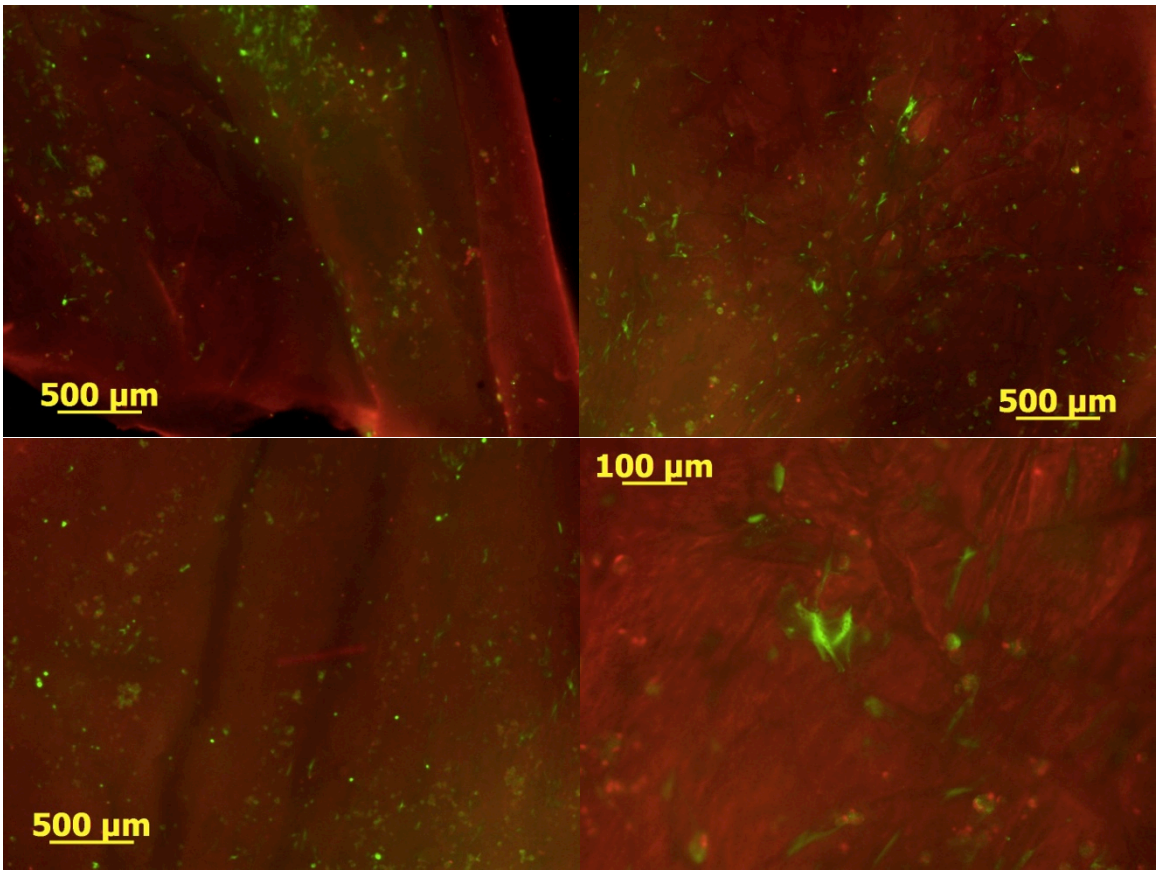


Figure 49: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation Live/Dead (Non-Coronary Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

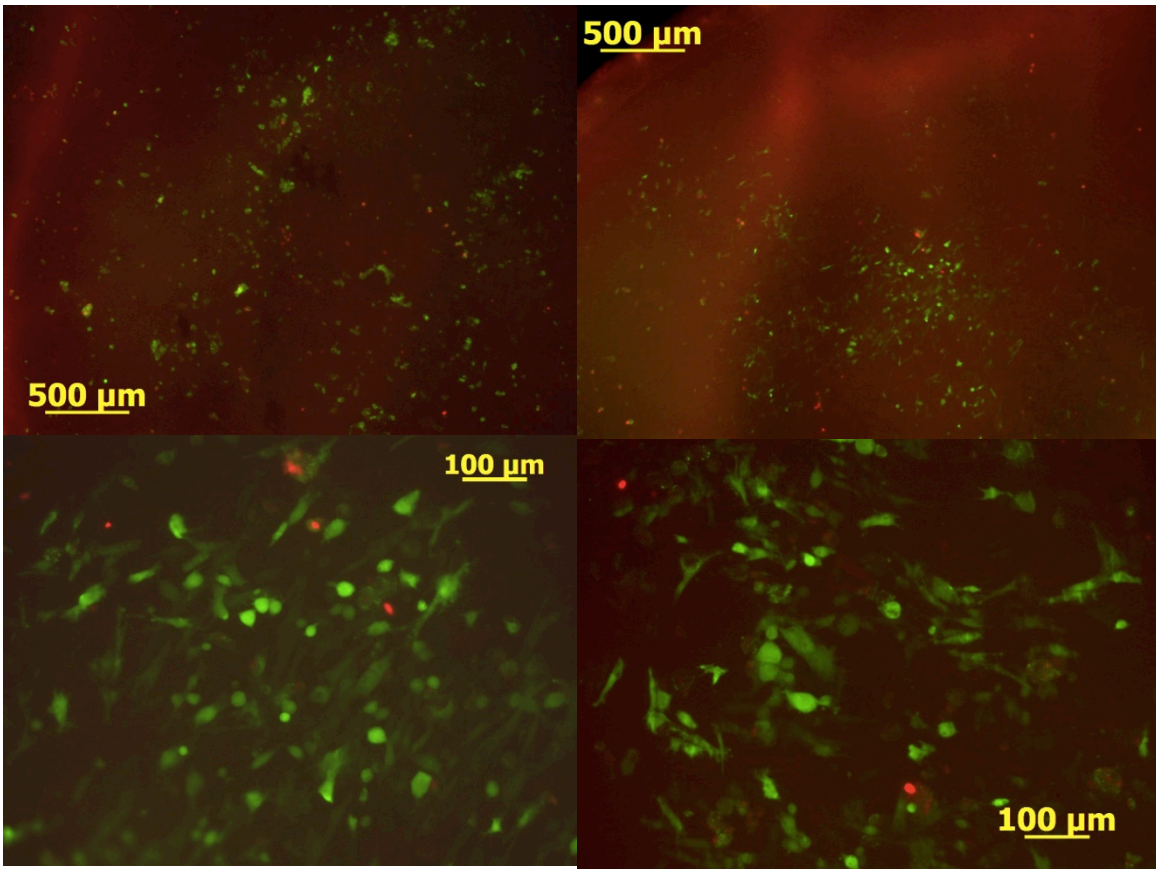


Figure 50: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation Live/Dead (Non-Coronary Ventricularis). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

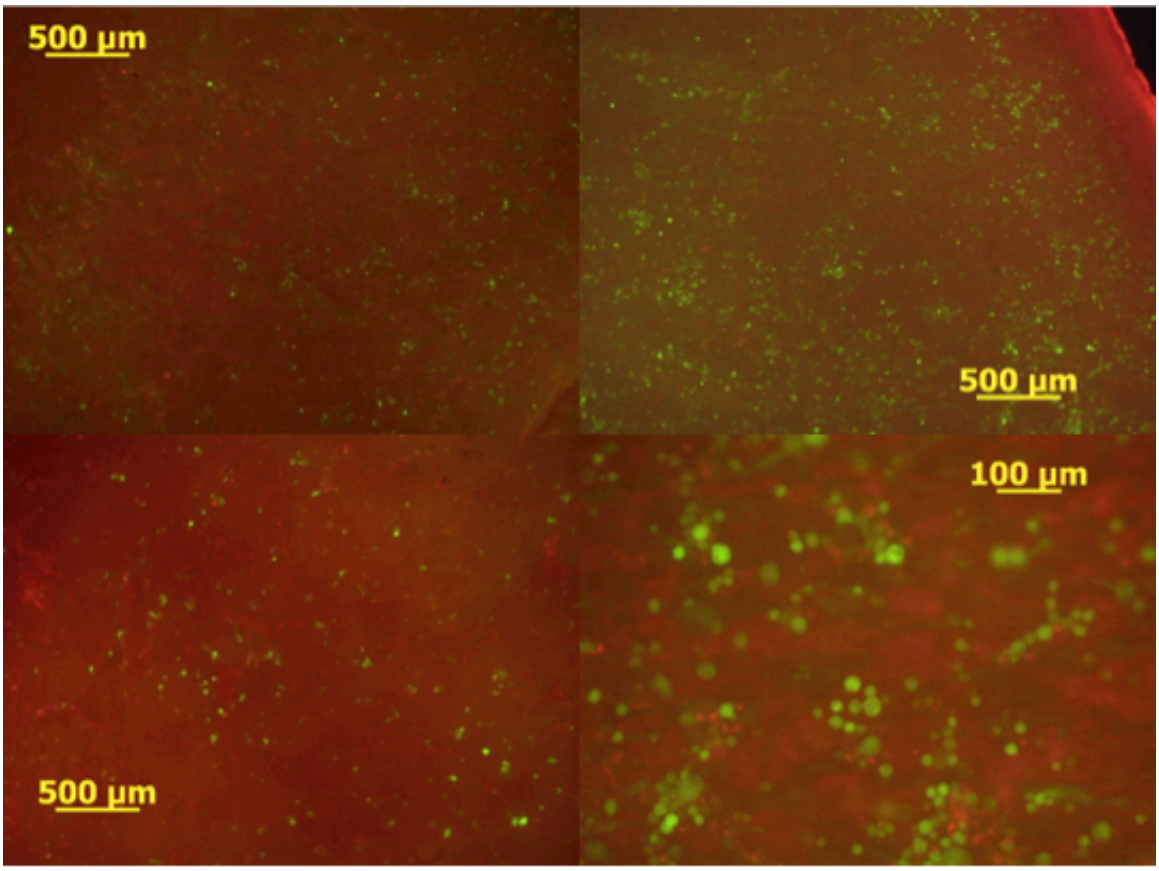


Figure 51: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation Live/Dead (Aortic Sinus). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

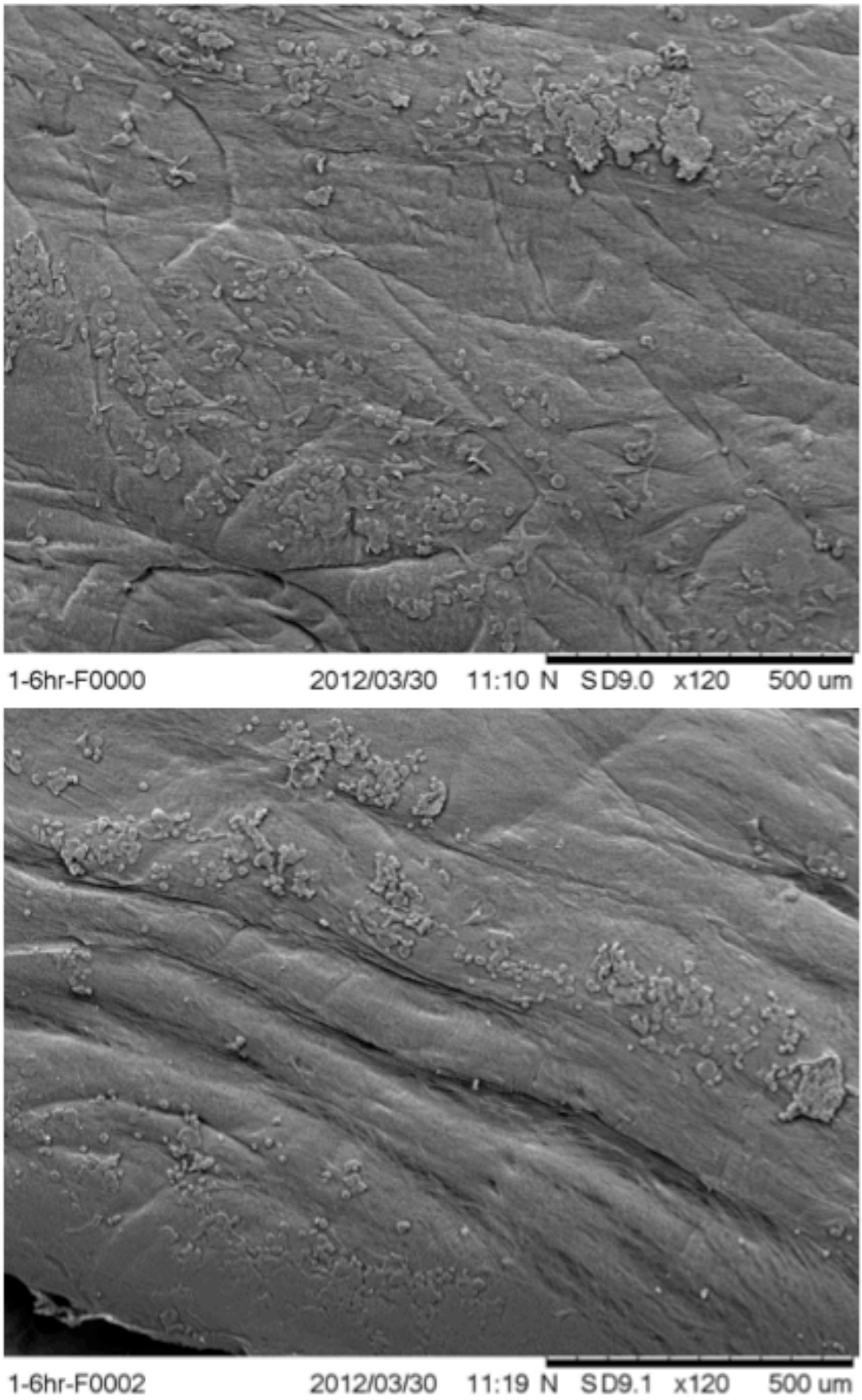


Figure 52: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation SEM (Fibrosa)

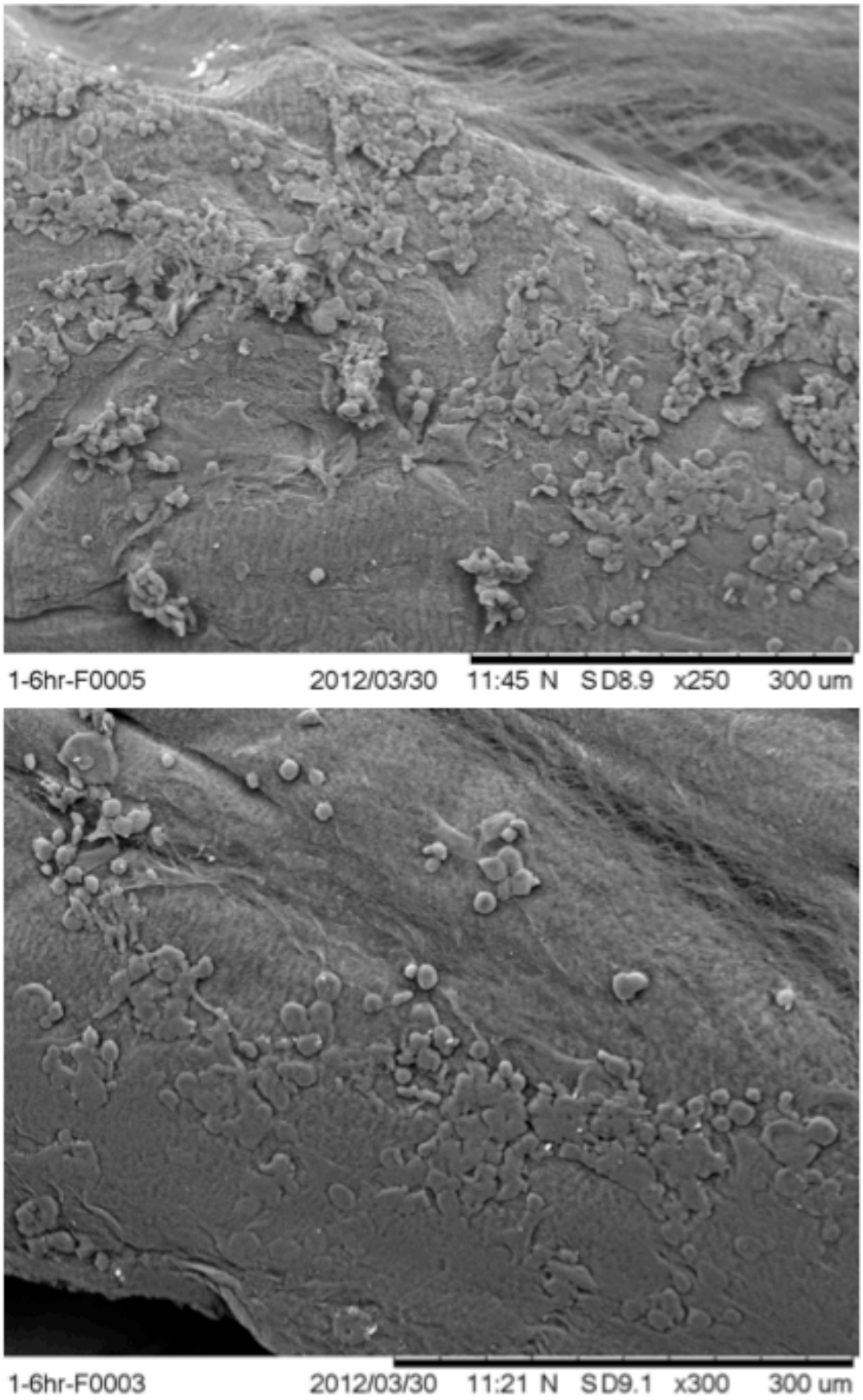


Figure 53: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation SEM (Fibrosa)

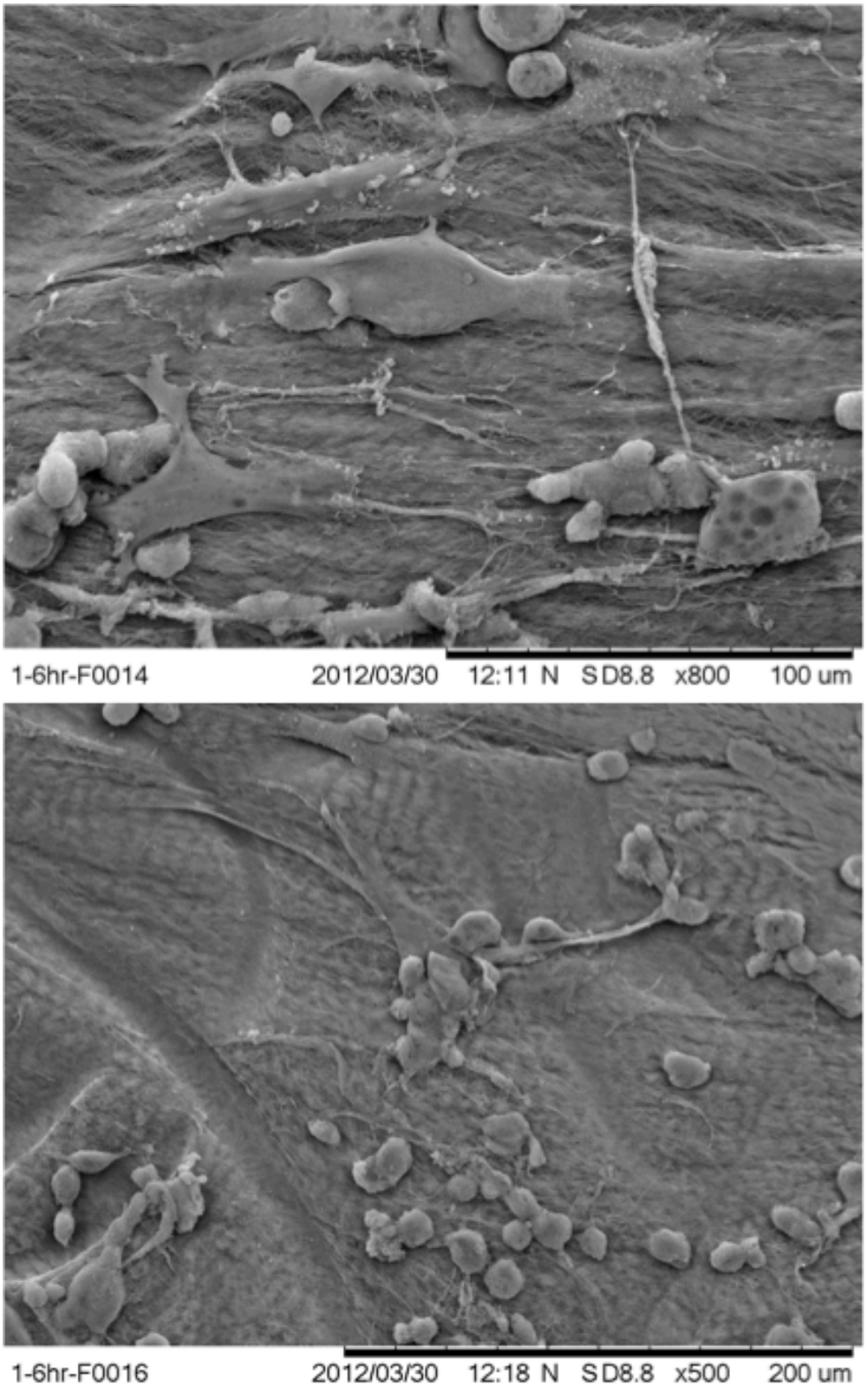


Figure 54: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation SEM (Fibrosa)

Figure 55: Study 5: 6 Hour Dynamic Seeding and 1 Hour Static Incubation SEM (Fibrosa)

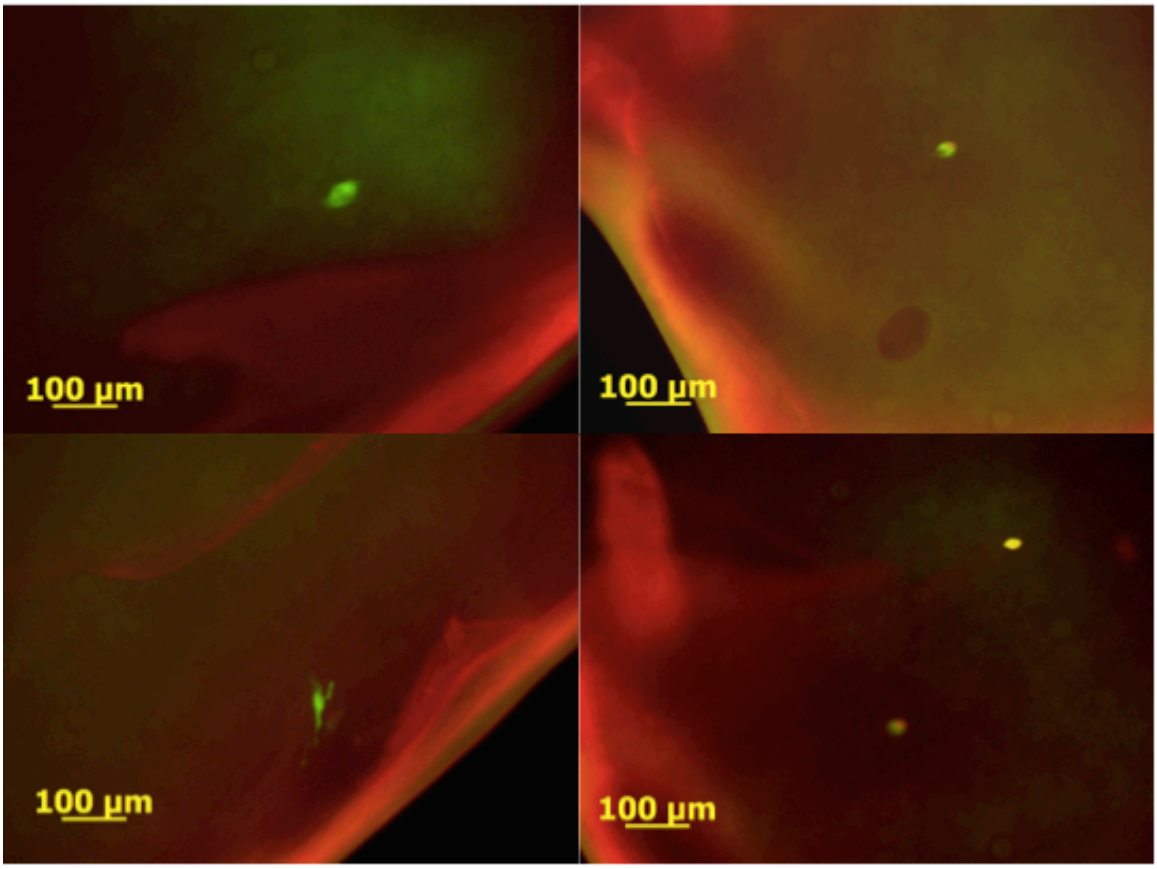


Figure 56: Study 5: Post 1 Week Mechanical Conditioning Live/Dead (Non-Muscle Coronary). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

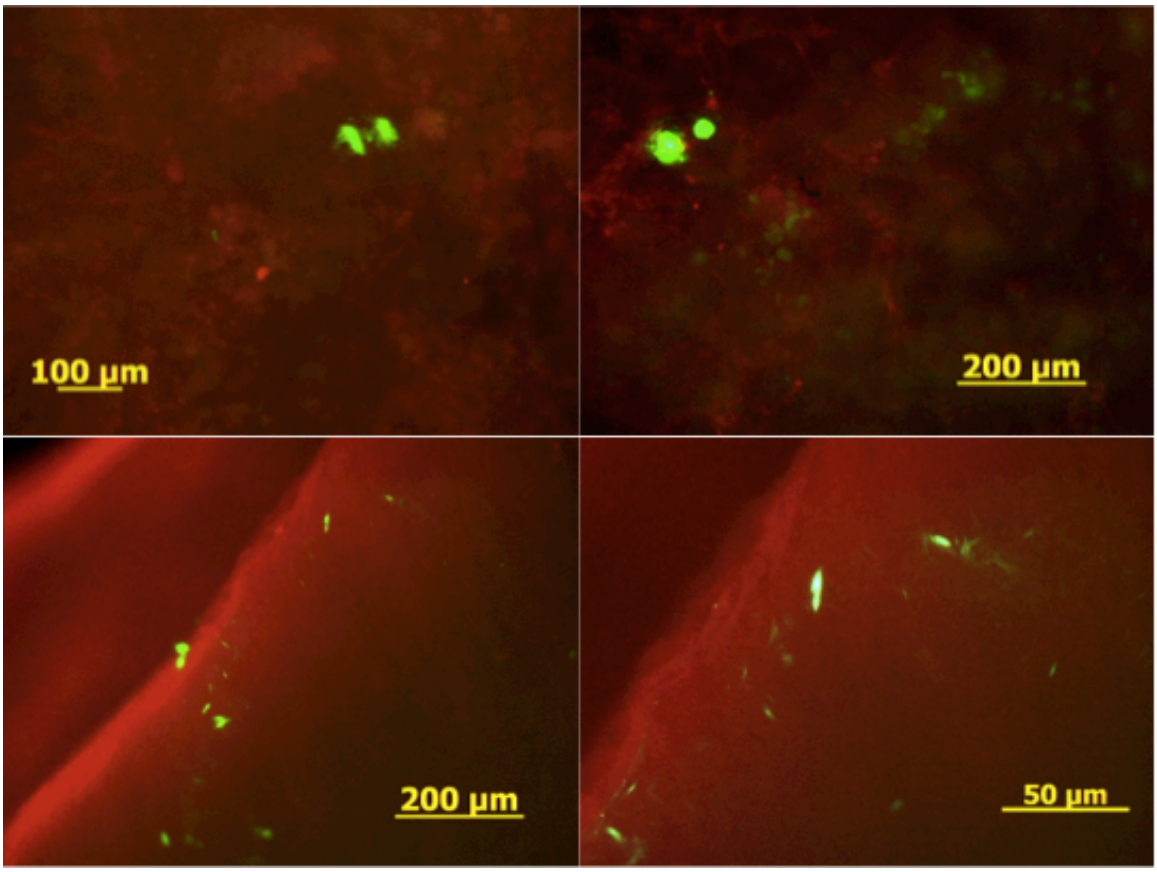


Figure 57: Study 5: Post 1 Week Mechanical Conditioning Live/Dead (Muscle Coronary). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

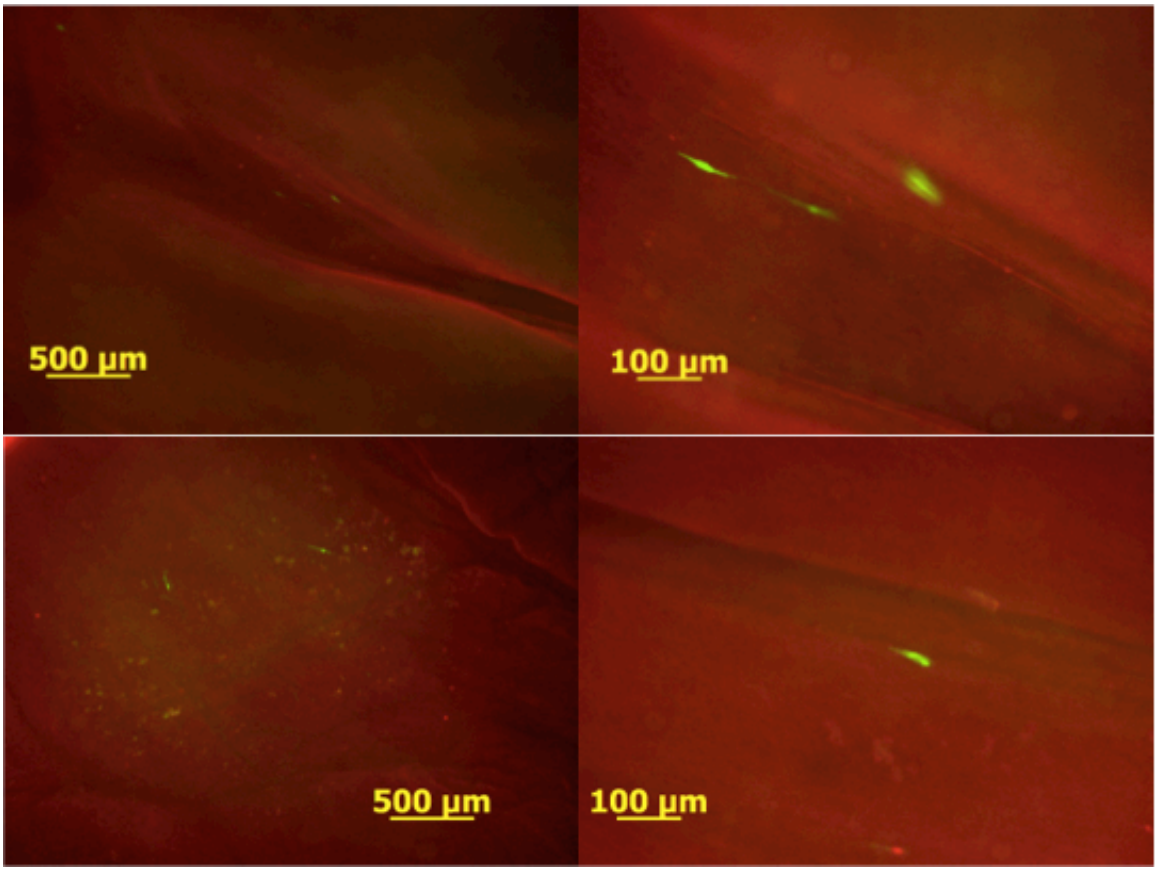


Figure 58: Study 5: Post 1 Week Mechanical Conditioning Live/Dead (Non-Coronary). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

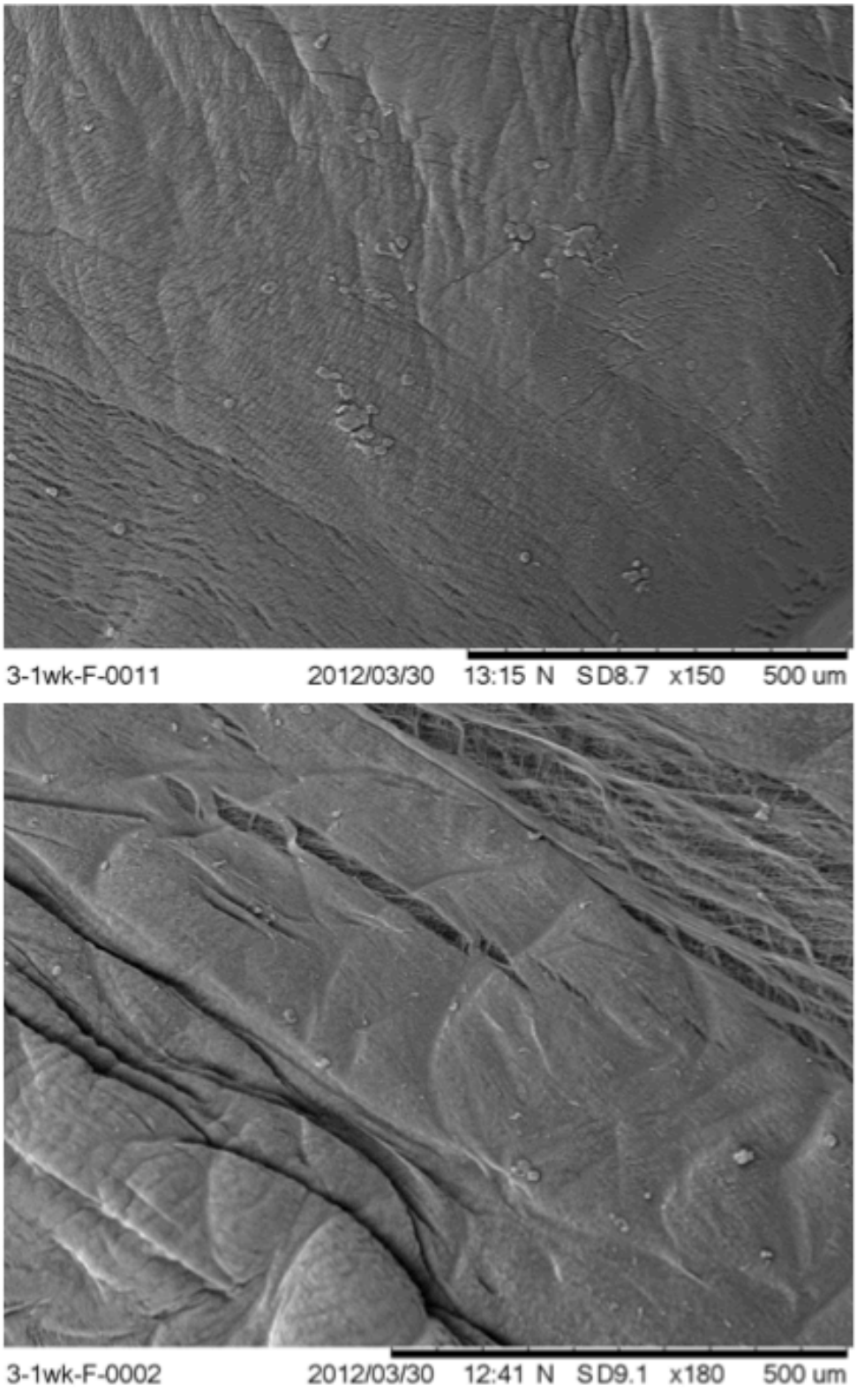


Figure 59: Study 5: Post 1 Week Mechanical Conditioning SEM (Fibrosa)

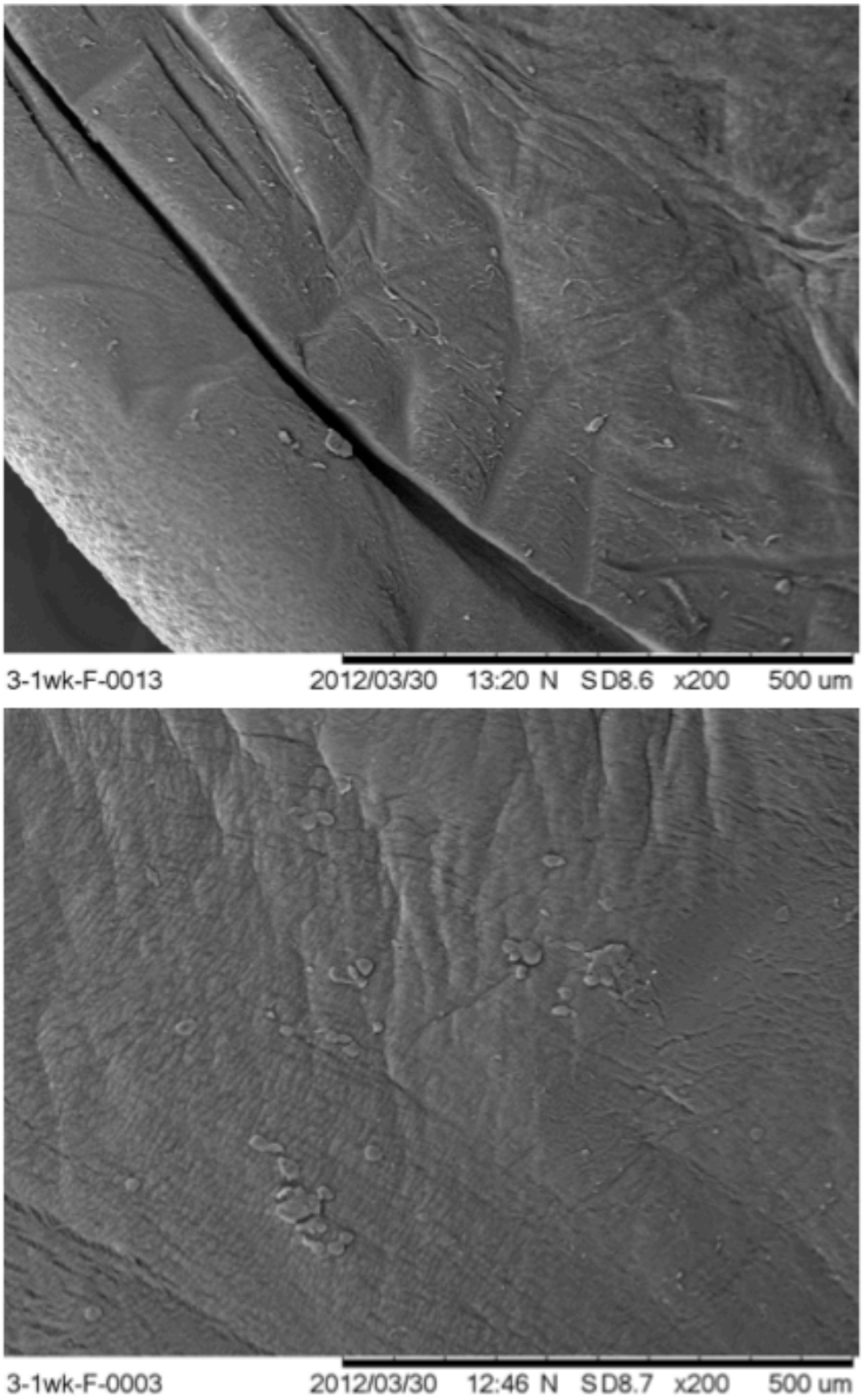


Figure 60: Study 5: Post 1 Week Mechanical Conditioning SEM (Fibrosa)

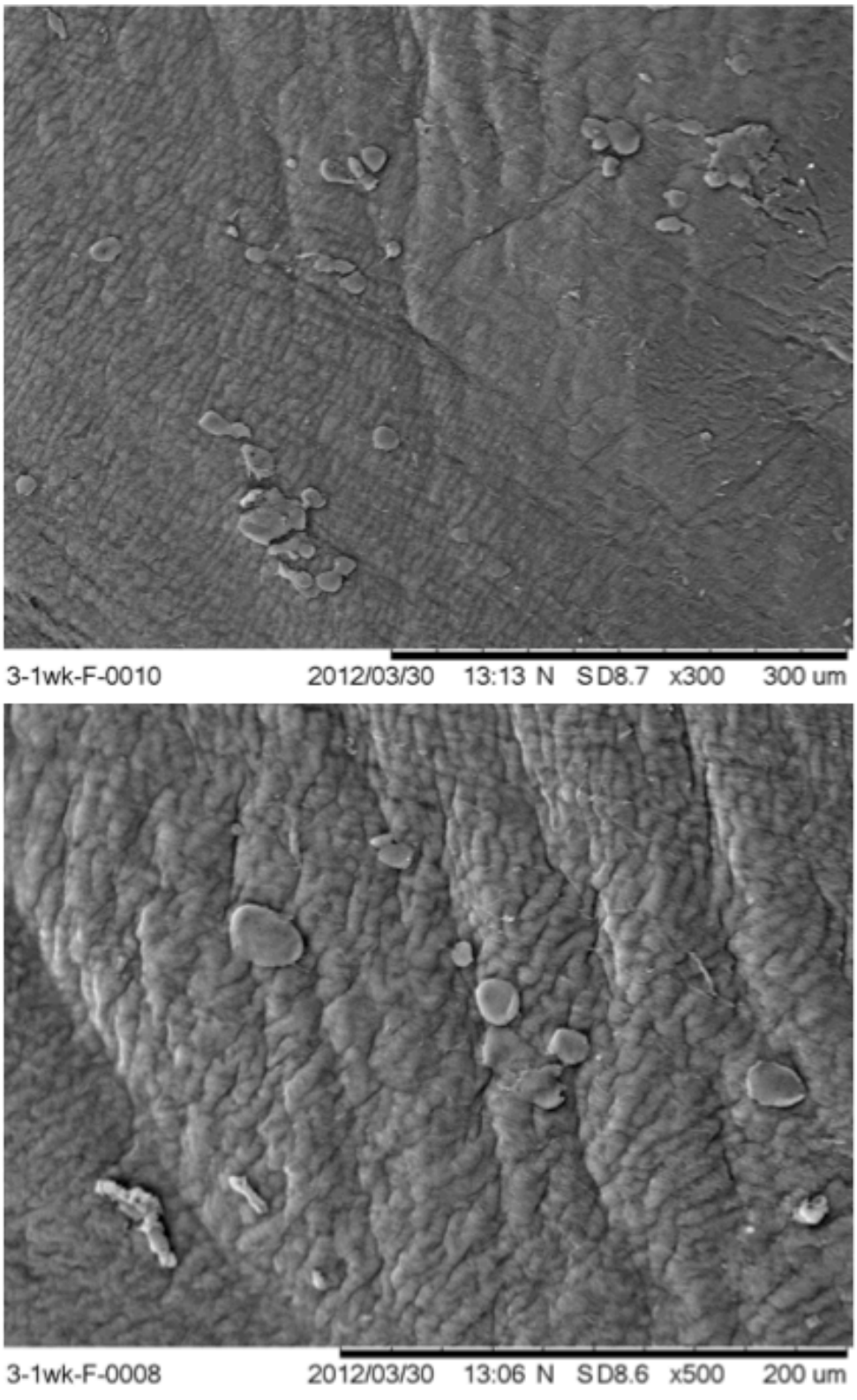


Figure 61: Study 5: Post 1 Week Mechanical Conditioning SEM (Fibrosa)

4.6 Study 6: Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions

The Live/Dead from images from the post 6 hour seeding and overnight incubation didn't indicate any live cells present (Figure 62). Based on previous results, the lack of cells on the scaffold didn't make much sense. Upon further observation, in place of green fluorescing live cells, green crystals were seen in images. It appeared that the live stain was contaminated or had gone bad. To investigate further the presence of cells, the scaffold was stained with DAPI and imaged (Invitrogen Eugene, OR). The DAPI stains cell nuclei blue and the results indicated that there were in fact numerous cells on the scaffold (Figure 62). Since the Dead stain was working and cells can be seen fluorescing red, the cells that appeared after DAPI stain were assumed to be alive. SEM imaging confirmed the presence of cells on the seeded scaffold (Figure 63 &Figure 64).

Analyzing the 2 week bioreactor conditioned scaffold through Live/Dead and SEM many cells were seen on the surface (Figure 65-Figure 75). The morphology of the present cells was of particular interest. Both the Live/Dead and SEM indicated the cells had spread from their rounded shape to an elongated shape and groups of cells appeared to be aligning with one another forming patterns similar to those seen in endothelial tissue (Figure 65-Figure 75). The mechanical conditioning clearly elicited a response in the cells on the scaffold causing them to elongate and align. These results indicated that using the dynamic seeding device, endothelial cells could effectively be seeded on the surface of a porcine decellularized PGG stabilized heart valve scaffold. In addition, following seeding, the use of the BTRL developed pulsatile heart valve bioreactor, these

cells could be retained alive on that surface and influence cell morphology under mechanical stimulation over a 2 week span. These results also indicate that the developed scaffold was friendly to cellular adhesion and viability.

Table 3: Pressure Values Over 2-week Bioreactor Retention Study

Date	Time	mmHg
3-Mar	1:00	15/15
	2:20	17/15
	6:00	23/15
	8:50	20/12
4-Mar	13:00	30/20
	15:15	35/23
5-Mar	15:15	40/25
6-Mar		40/25
7-Mar		40/25
8-Mar		40/25
9-Mar		40/25
10-Mar		40/25
11-Mar		40/25
12-Mar		40/25
13-Mar		40/25
14-Mar		40/25
15-Mar		40/25
16-Mar	12:45	Removed

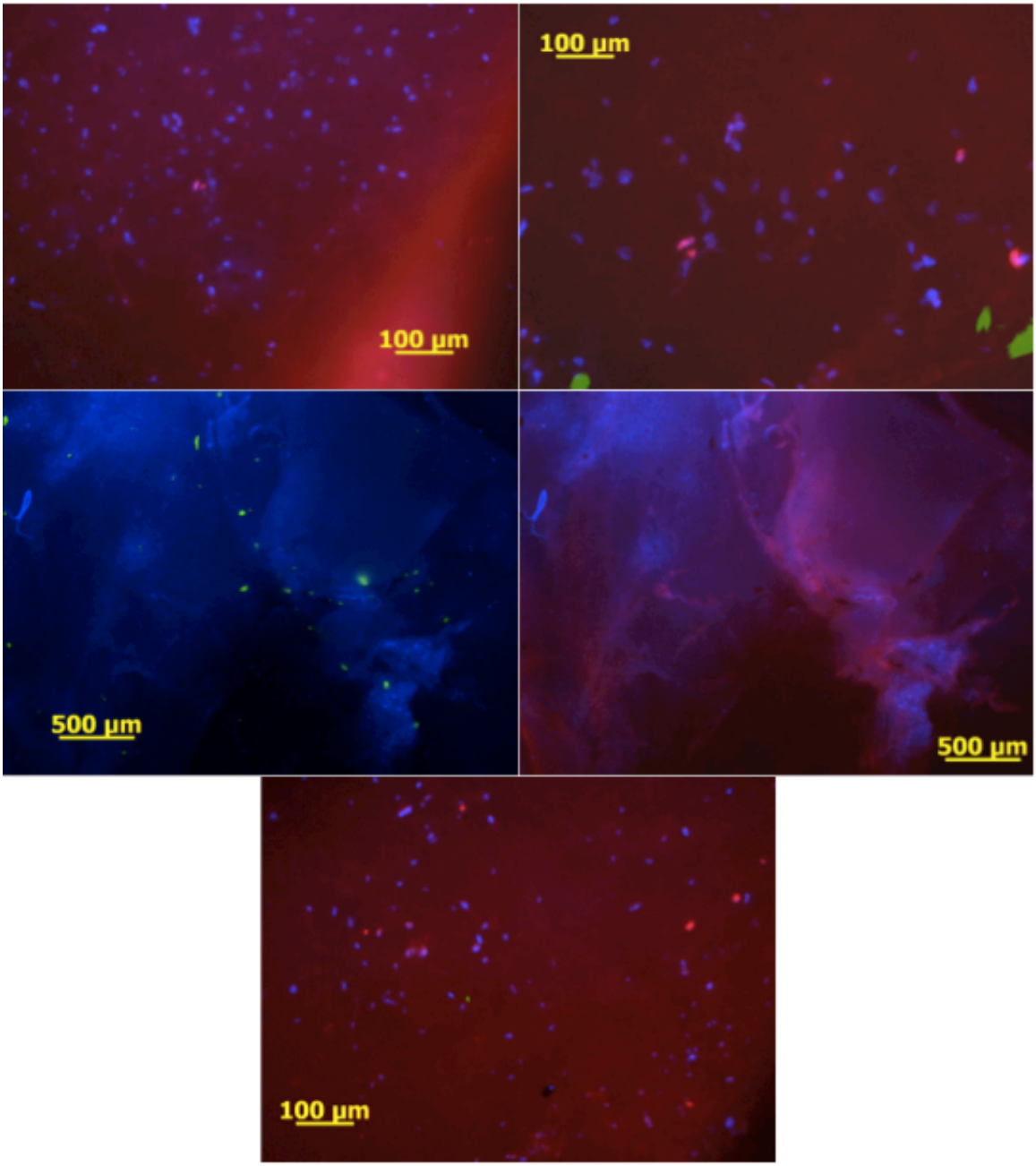
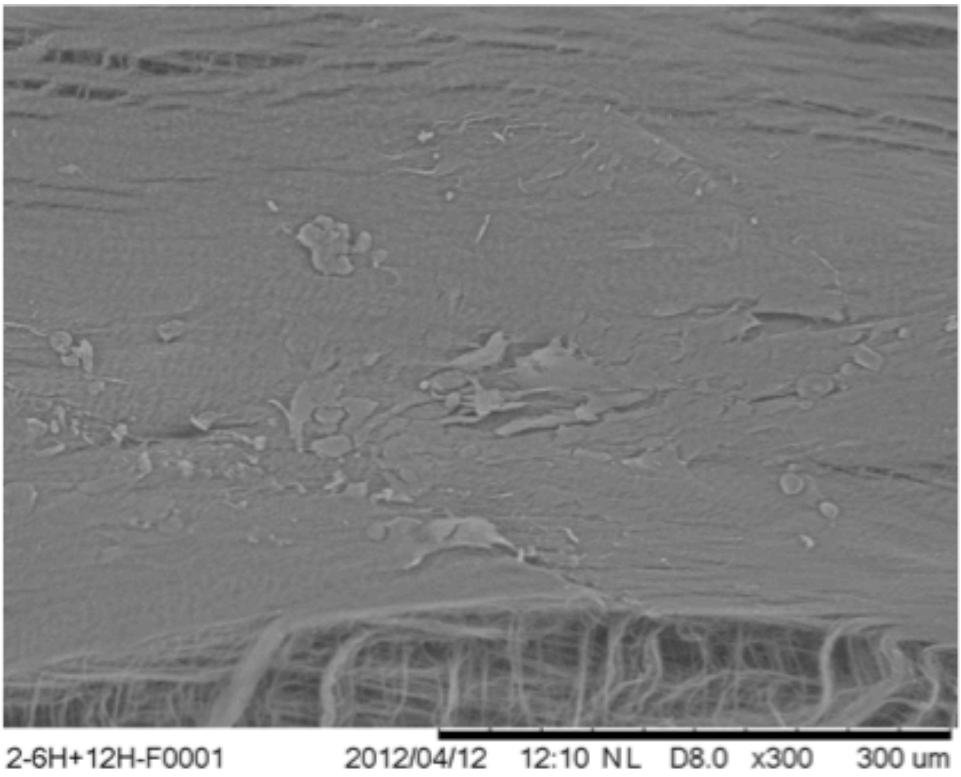


Figure 62: Study 6: Post 6 Hour Dynamic Seeding and Overnight Static Incubation Live/Dead + DAPI. Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red. DAPI Stain Binds DNA and Cell Nuclei Fluoresce Blue



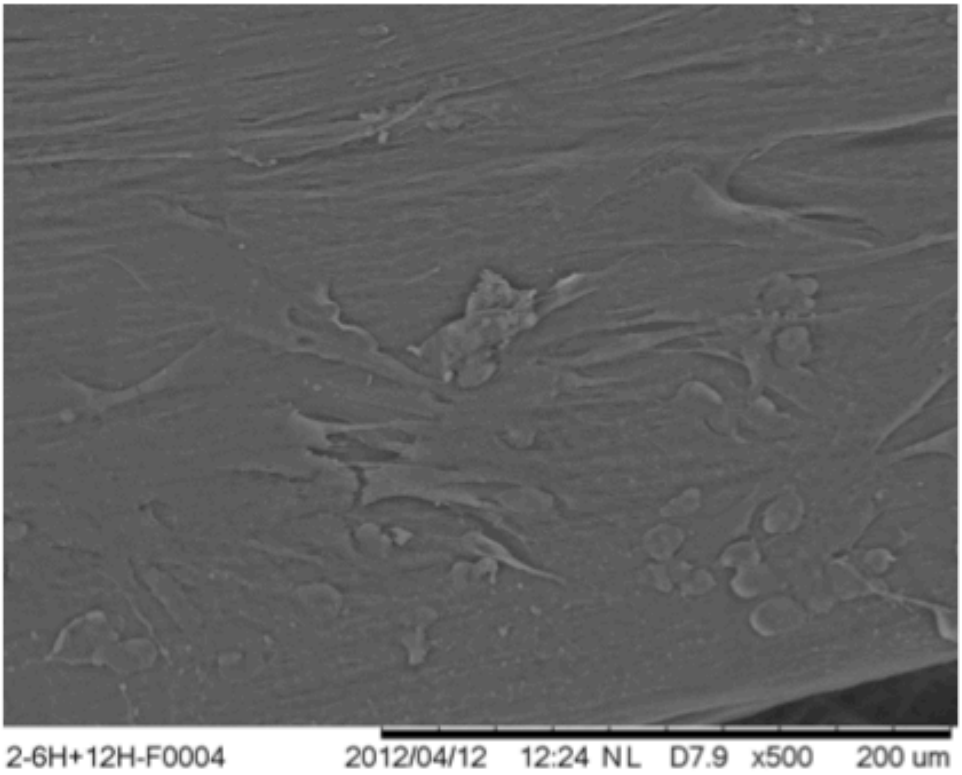


Figure 63: Study 6: Post 6 Hour Dynamic Seeding and Overnight Static Incubation SEM (Fibrosa)

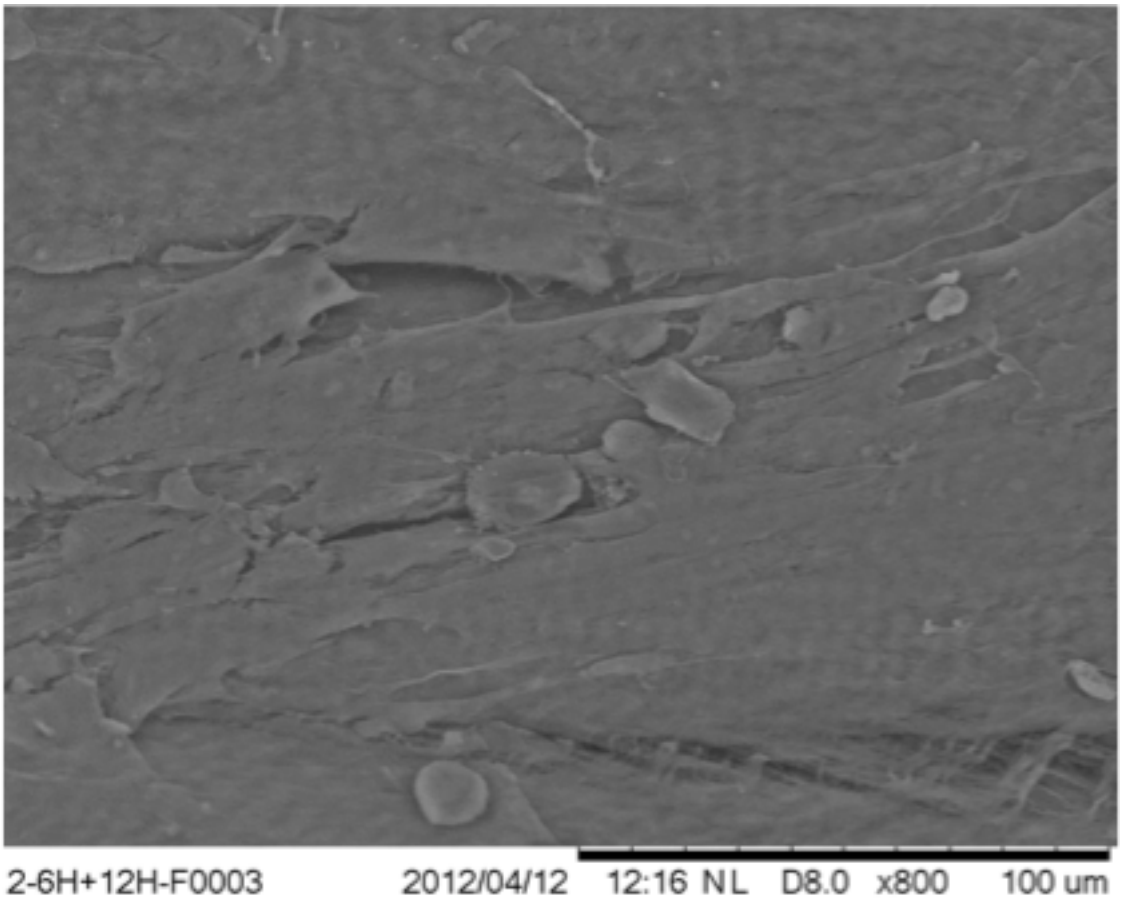


Figure 64: Study 6: Post 6 Hour Dynamic Seeding and Overnight Static Incubation SEM (Fibrosa)

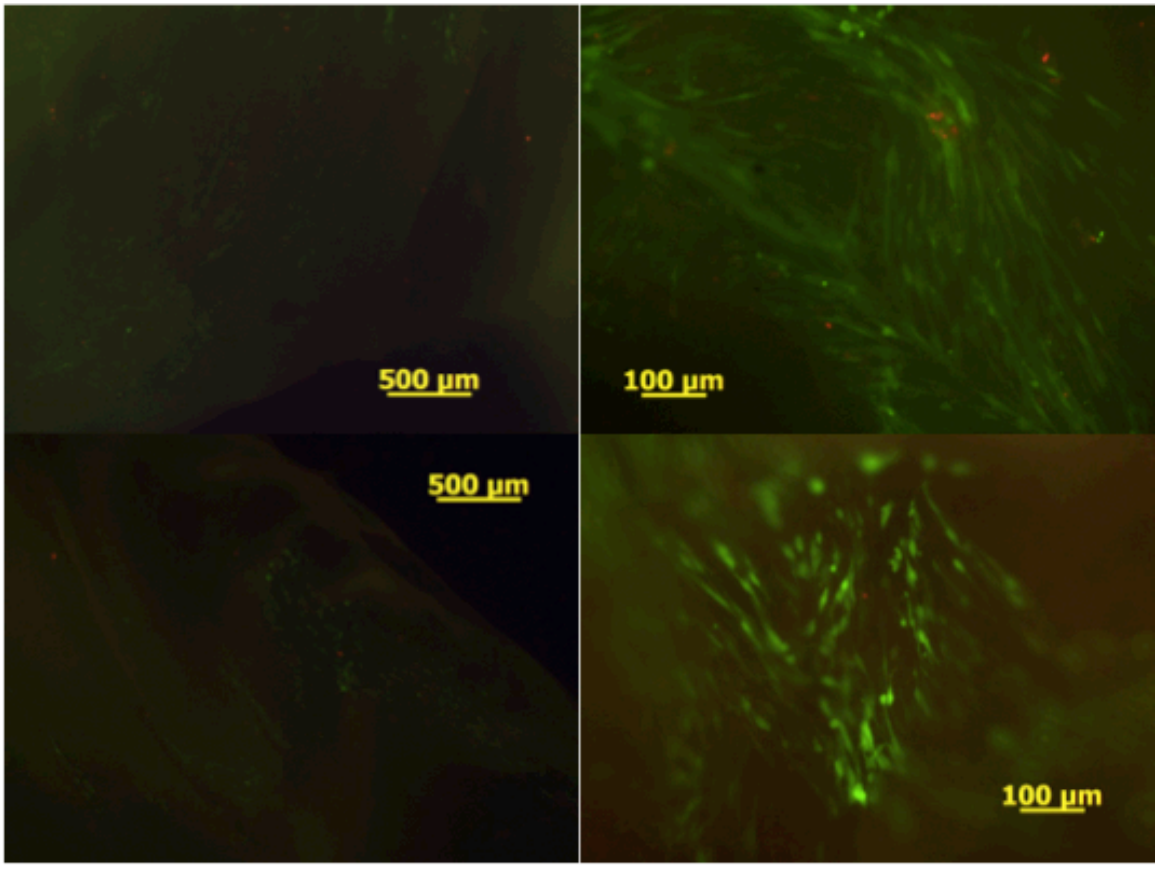


Figure 65: Study 6: Post 2 Week Mechanical Conditioning Live/Dead (Fibrosa). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

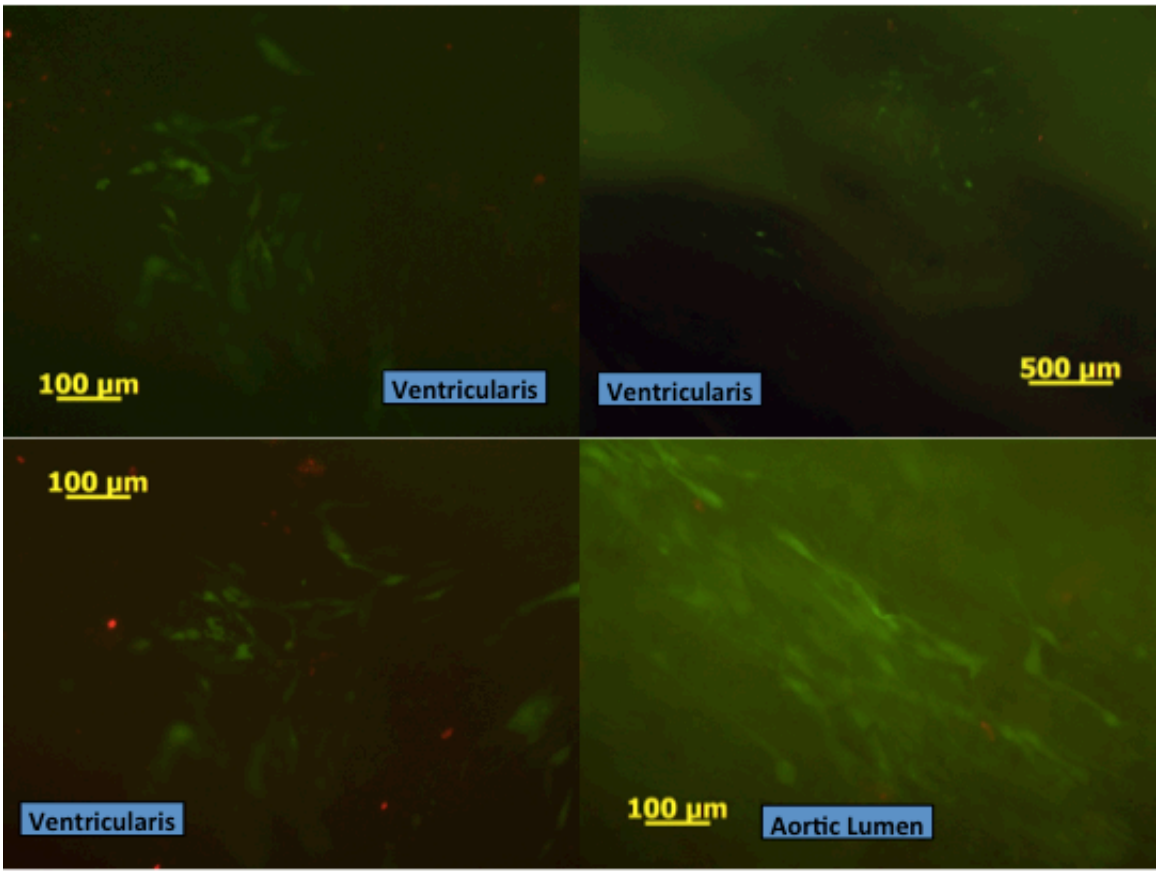


Figure 66: Study 6: Post 2 Week Mechanical Conditioning Live/Dead (Ventricularis & Aortic Lumen). Living Cells are Stained and Fluoresce Green. Dead Cells Fluoresce Red

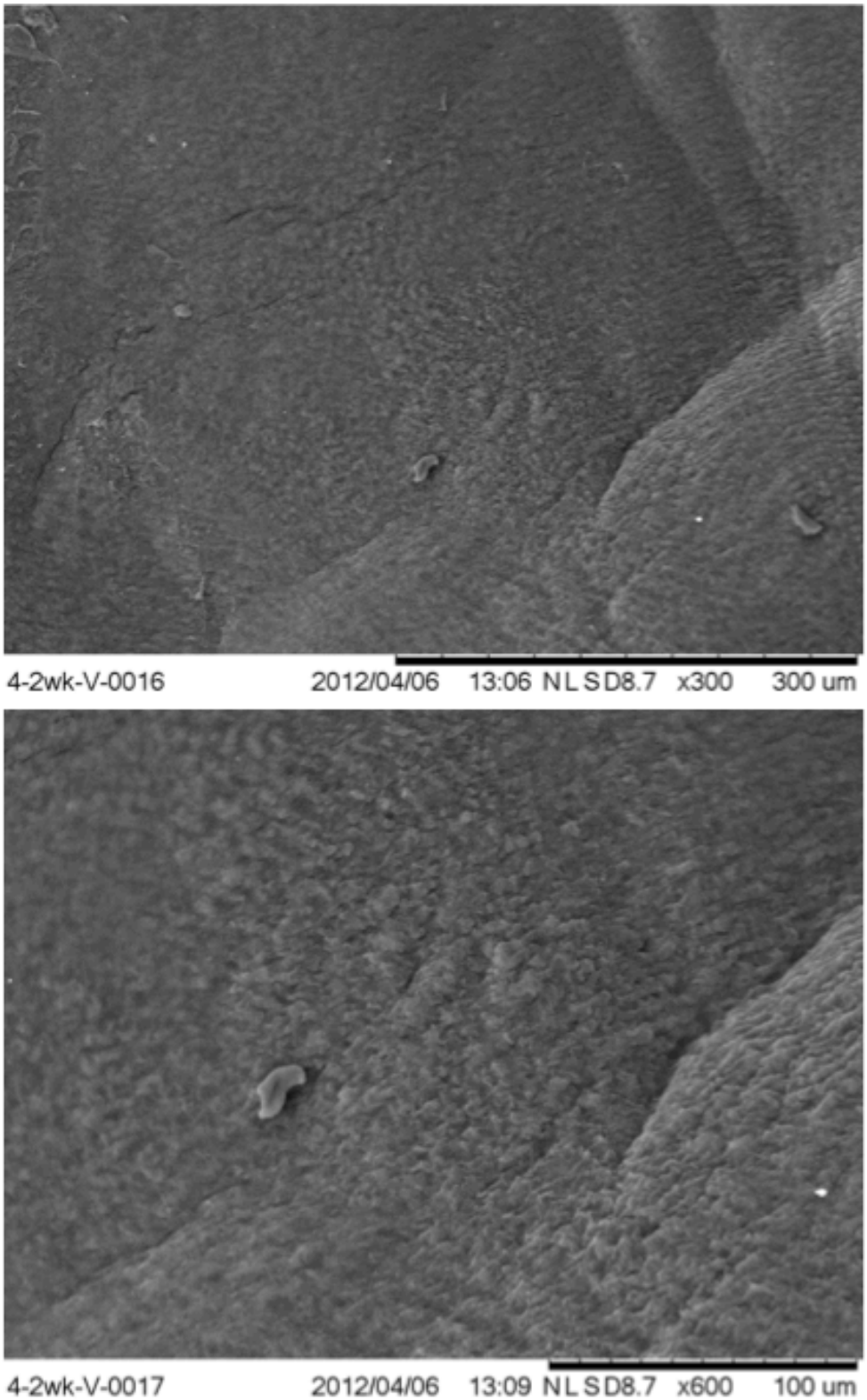


Figure 67: Study 6: Post 2 Week Mechanical Conditioning SEM (Ventricularis)

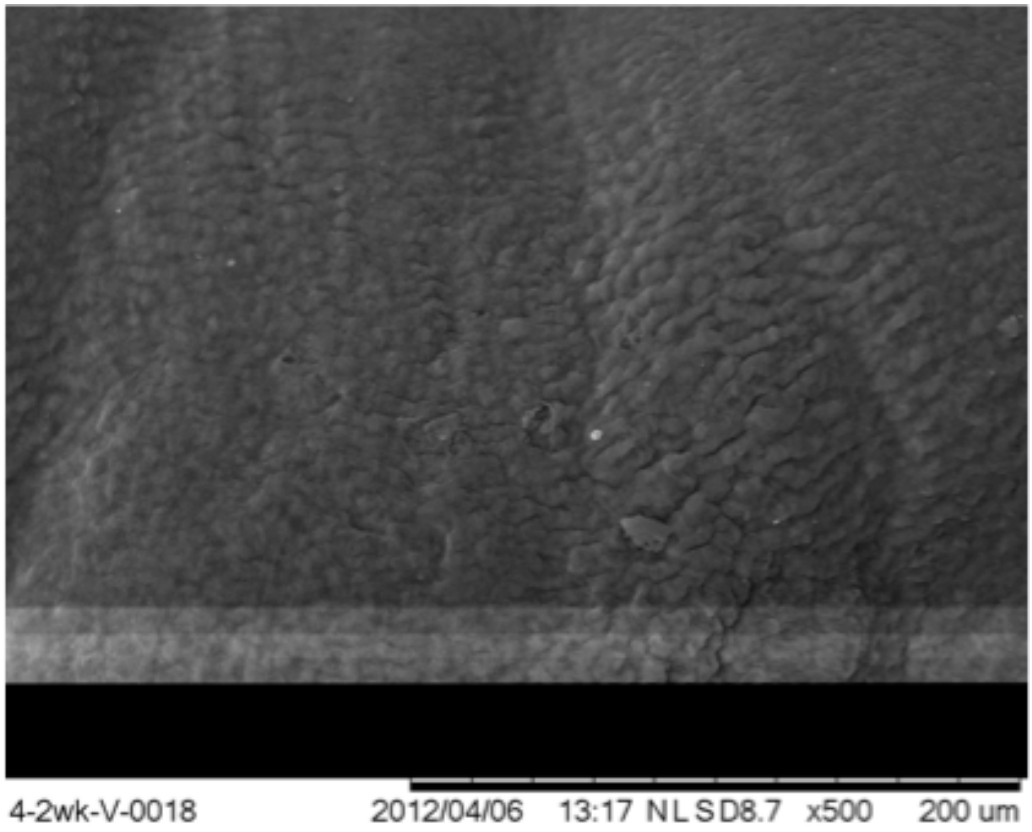


Figure 68: Study 6: Post 2 Week Mechanical Conditioning SEM (Ventricularis)

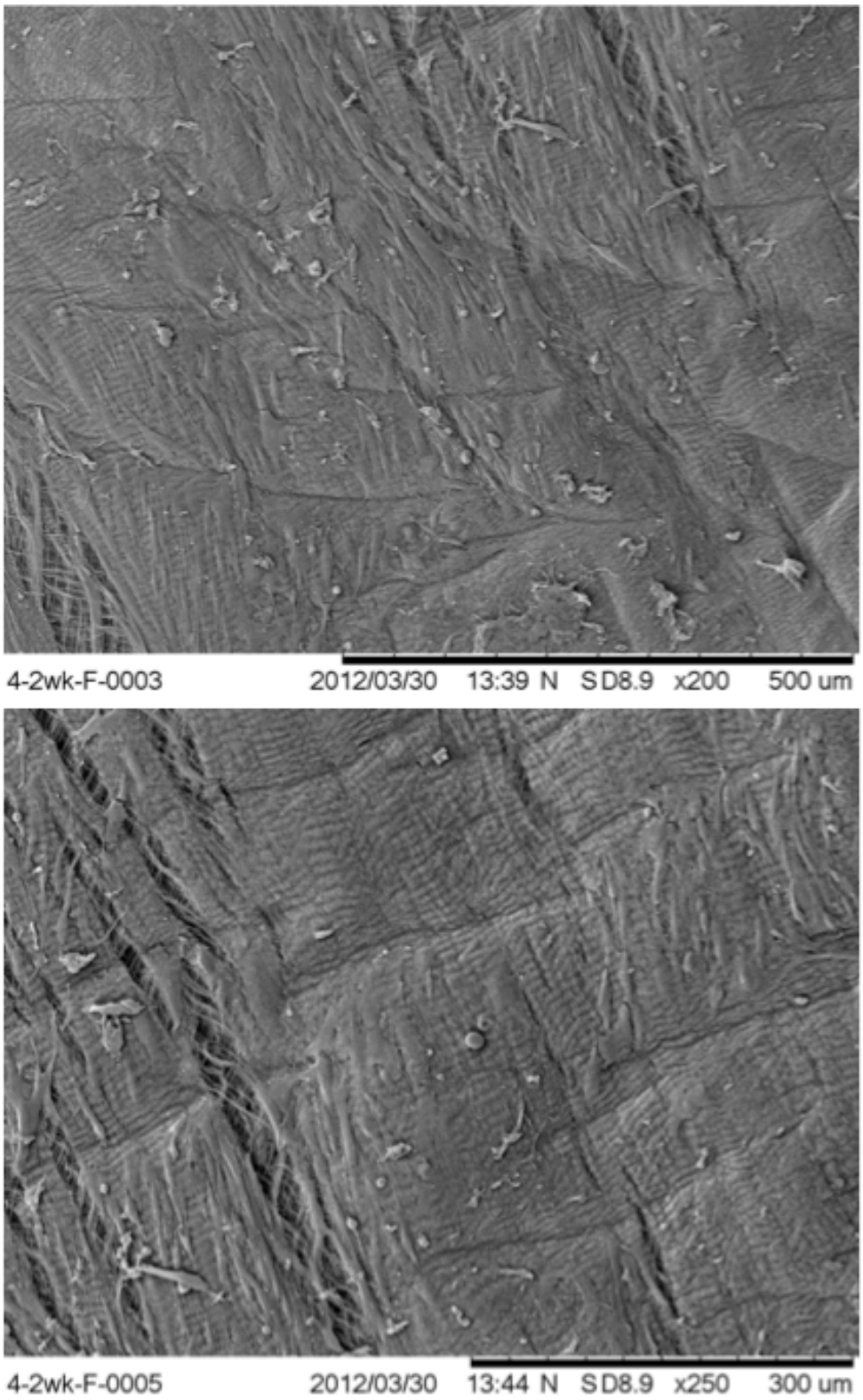


Figure 69: Study 6: Post 2 Week Mechanical Conditioning SEM (Fibrosa)

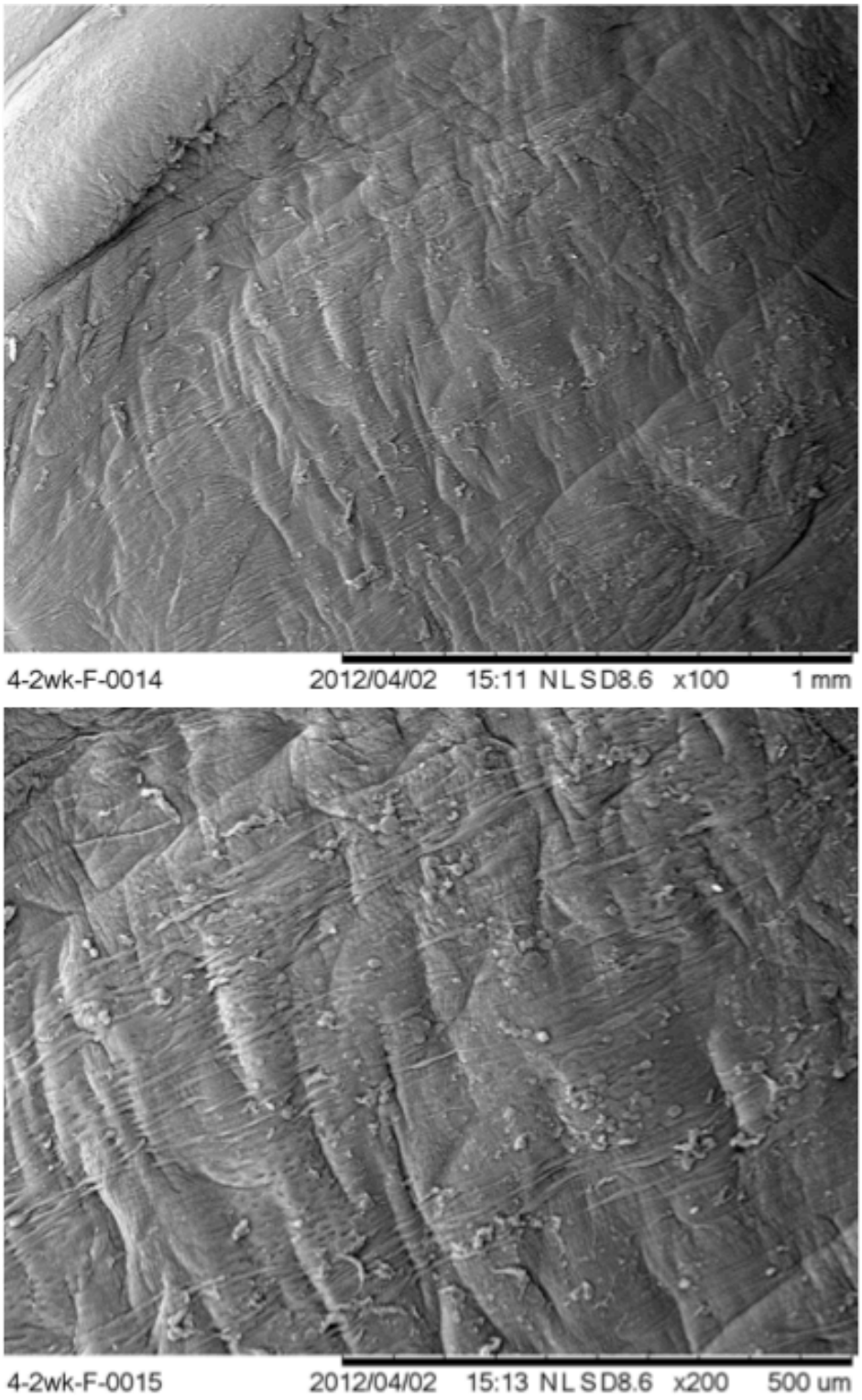


Figure 70: Study 6: Post 2 week Mechanical Conditioning SEM (Fibrosa)

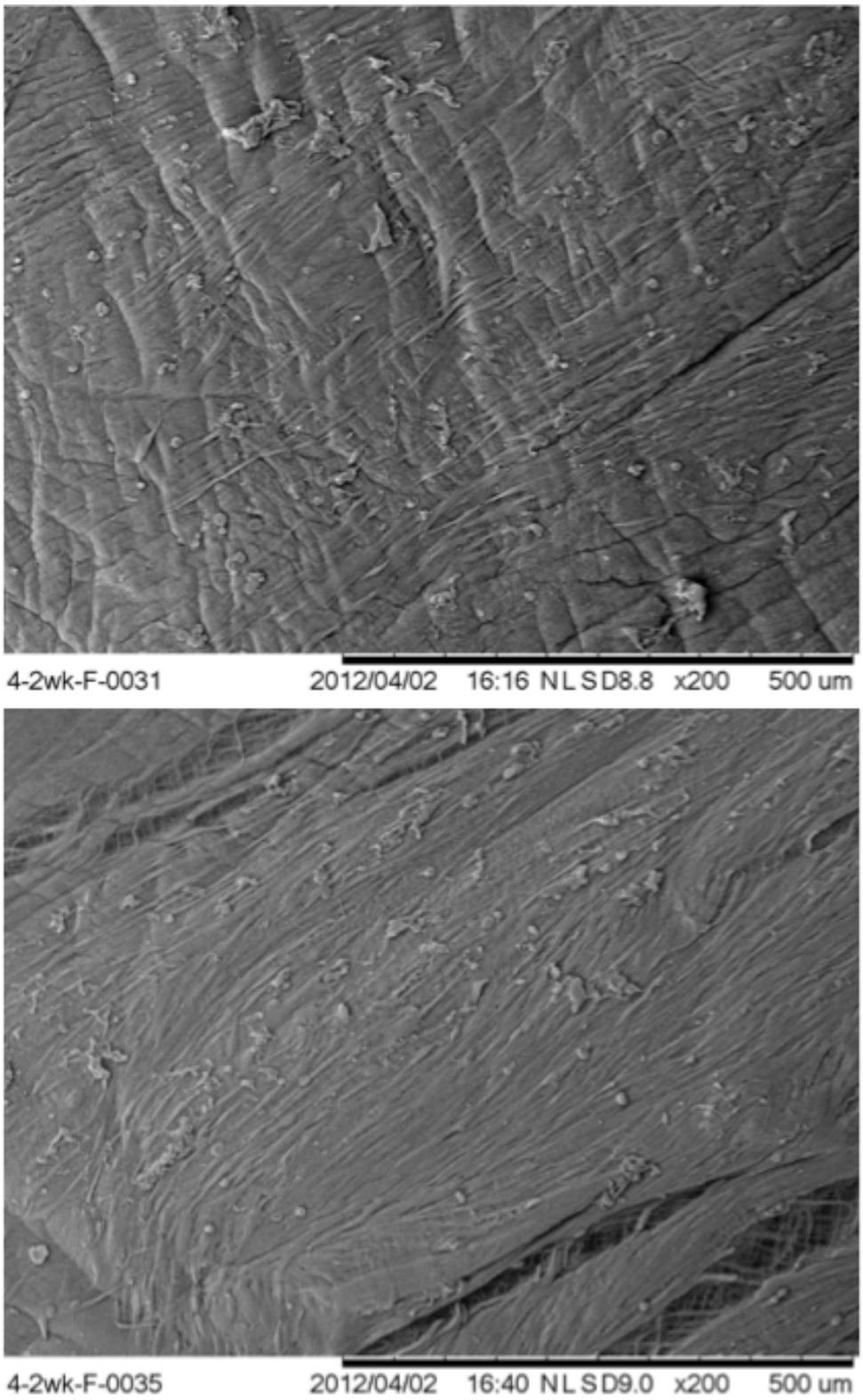


Figure 71: Study 6: Post 2 Week Mechanical Conditioning SEM (Fibrosa)

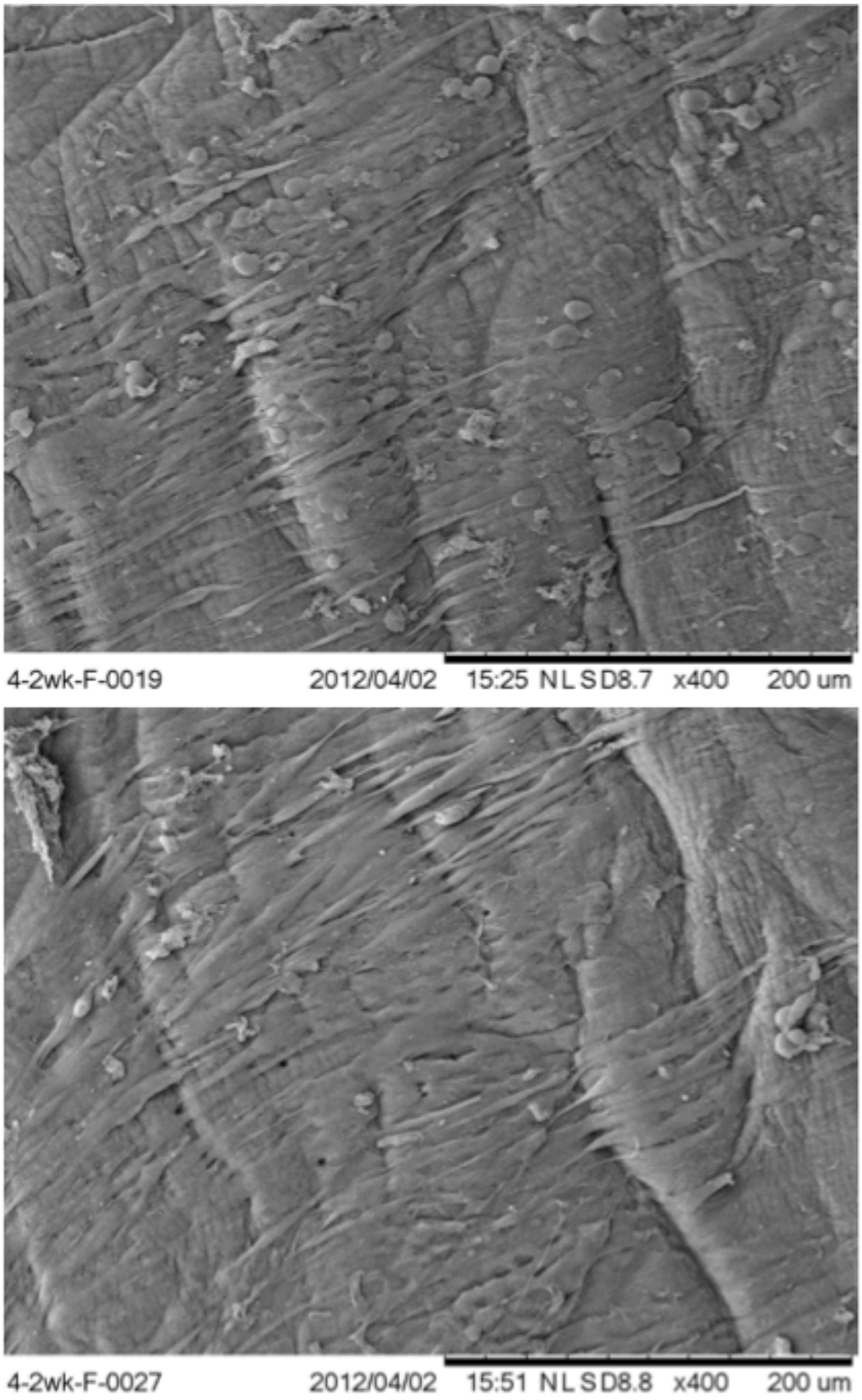


Figure 72: Study 6: Post 2 Week Mechanical Conditioning SEM (Fibrosa)

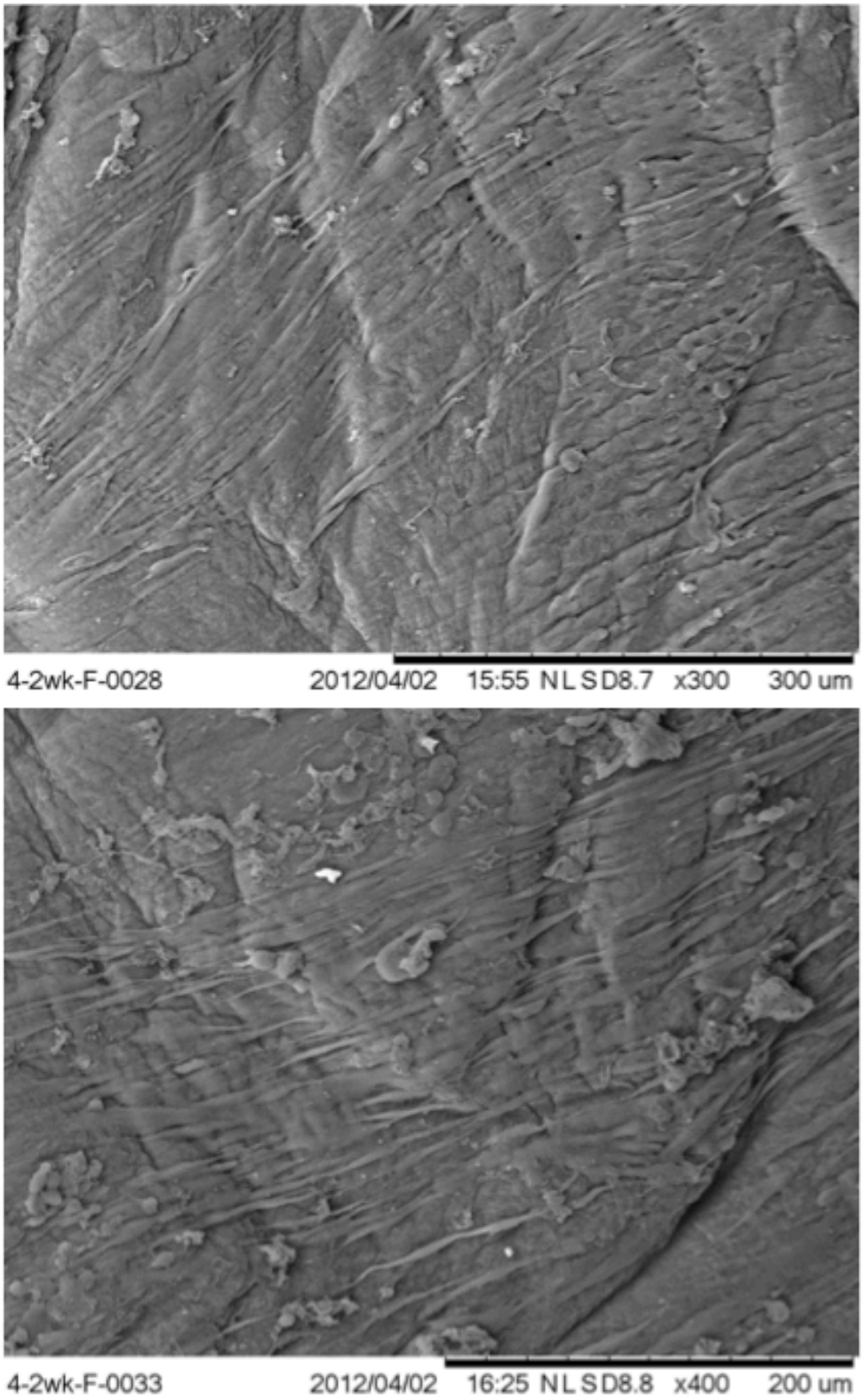


Figure 73: Study 6: Post 2 Week Mechanical Conditioning SEM (Fibrosa)

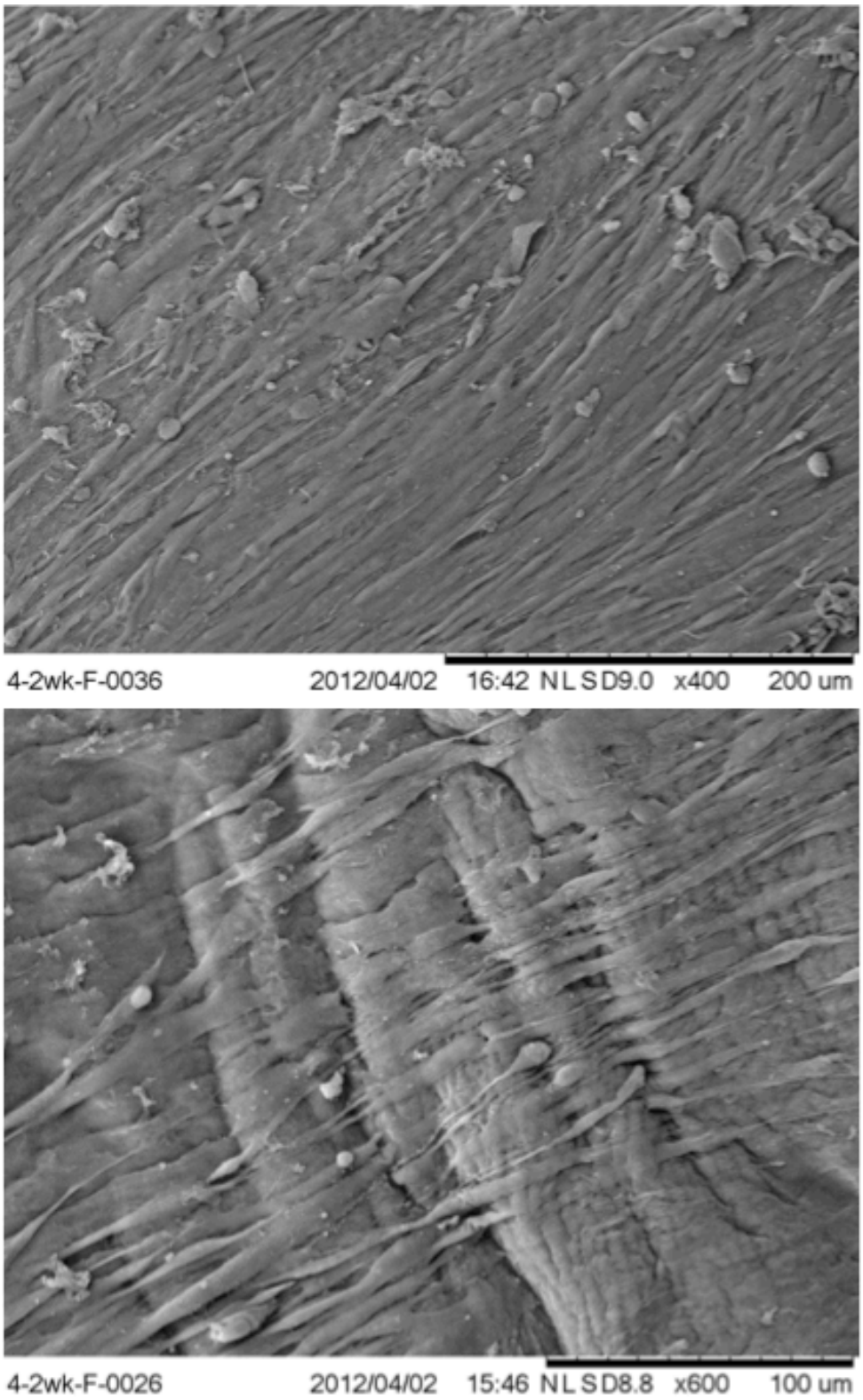


Figure 74: Study 6: Post 2 Week Mechanical Conditioning SEM (Fibrosa)

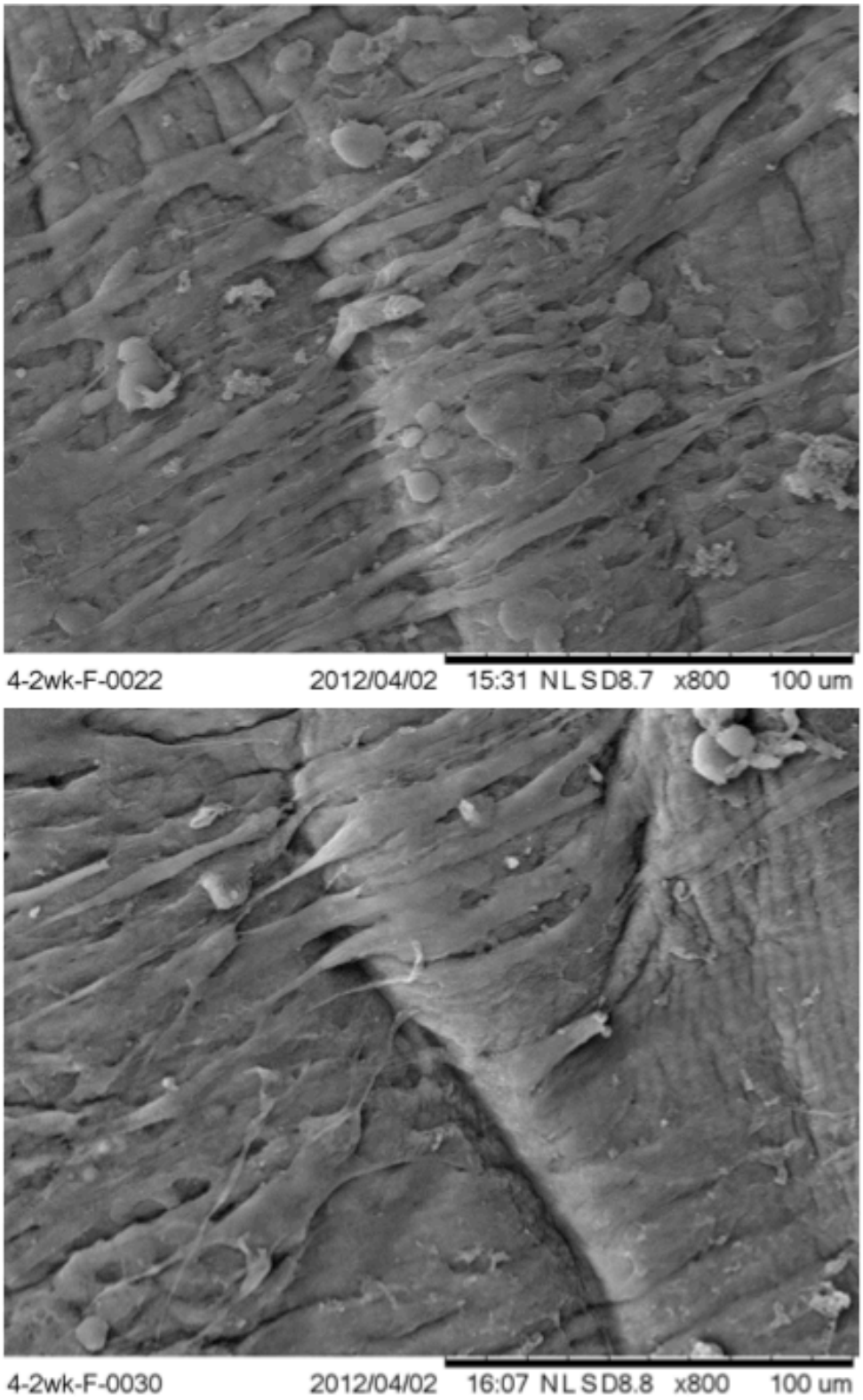


Figure 75: Study 6: Post 2 Week Mechanical Conditioning SEM (Fibrosa)

CONCLUSIONS

5.1 Study 1: Initial Examination of Design Concept and Establishment of General Parameters (Black Pepper Study)

- Through the use of pepper as a visible representation of cells, the flow of fluid both around and through a mounted decellularized heart valve scaffold was visually observed.
- The pepper came into contact with all surfaces of the valve supporting the device's ability to facilitate cell to surface contact during seeding experiments.
- An ideal suspension volume, axis of rotation, and speed was determined from this expeiement.

5.2 Study 2: Initial Low Concentration Cell-Seeding Study

- After 2 hours of dynamic cell-seeding the device, living cells were observed to be present on all surfaces of a decellularized heart valve scaffold including the fibrosa, ventricularis, coronary sinus, and adventitia and lumen of the aorta.
- The device was demonstrated to facilitate cell to surface contact and subsequent cellular adherence.
- The patterned one-sided attachment noted on the non-axial cusps lead to the modification of the protocol to incorporate a static interval and a 120 degree turn of the chamber for future studies.
- Only 18% of cells remained in the cellular suspension after the study, suggesting a high efficiency of initial cell attachment.

5.3 Study 3: Higher Concentration Cell-Seeding Study: Focusing on Increasing Coverage Uniformity

- The additional steps to the protocol yielded more uniform cellular coverage and eliminated patterning.
- The variability in coverage lead to the adoption of more uniform experimental control for future studies utilizing computer software, a microcontroller, and stepper motor.
- 38% of the cells remained in suspension which lead to the incorporation of longer seeding times into future studies with the aim of facilitating higher cellular attachment.

5.4 Study 4: Computer Control For Increased Coverage and Variable Seeding-Time Study

- Computer control normalized the procedure making it more repeatable while eliminating human factors.
- Results indicated that cells could remain viable under a 6 hour seeding step and that the increased time over 2 and 4 hour samples also facilitated increased cellular attachment.

5.5 Study 5: Dynamic Seeding with 1 Week Heart Valve Bioreactor Cellular Retention Under Systemic Conditions

- Post 6 hour dynamic seeding, cells were seen present on all surfaces of the decellularized heart valve scaffold.

- After 1 week dynamic conditioning under systemic pressures (120/80mmHg) and conditions, very few cells remained on the surface of the valve.
- The cells were likely washed away due to the high pressure and shear forces. As such the conclusion that a lower pressure or slower pressure ramp could mitigate the cellular detachment.

5.6 Study 6: Fibronectin Treated Scaffold, Dynamic Seeding, and 2 Week Heart Valve Bioreactor Under Pulmonary Conditions

- Post 6 hour dynamic seeding DAPI and SEM showed cells were successfully attached to the surface of the decellularized heart valve scaffold.
- Fibronectin and overnight static incubation increased cellular attachment and morphology of the cells was notably different appearing more spread across the surface and more securely attached.
- After 2 weeks in the bioreactor under pulmonary pressures (40/25mmHg) and conditions, the cell morphology had changed toward an elongated shape and groups of cells had aligned to cover the surface.
- More cells were present on the fibrosa surface than the ventricularis surface.

5.7 Final Conclusions and Recommendations for Future Studies

Conclusions

1. The seeding container allows for efficient fluid flow in and around the valve

2. Computer controlled 3-dimensional rotation of the valves in the orbital shaker/A-frame hybrid system yield improved cell seeding
3. Fibronectin coating of the scaffold improves cell adhesion
4. Progressive adaptation to increased pressures could insure optimal cell attachment and long term cell retention in pulmonary conditions

Recommendations

- Future studies need to focus on iterative optimization of dynamic seeding parameters using the current devices
- Focusing on the sequence of rotation/pause steps and the length and frequency of each
- Investigate longer static seeding times for promoting a stronger cell adhesion
- Possible addition of seeding steps after bioreactor conditioning
- Look into alternative cell removal techniques other than Trypsin such as cell scraping to remove cells from flasks while maintaining surface integrins
- Incorporate a more gradual ramping up pressures to reach arterial conditions in the bioreactor to reduce the possible loss of attached cells due to shear force and pressure
- Look into additional scaffold treatments that promote cellular adherence
- Look at the phenotype of endothelial cells post seeding utilizing CD-31
- Explore additional cell types and sources for seeding (Adipose-derived Stem Cells or Endothelial Progenitor Cells)

- Develop a method for seeding valvular interstitial cells (VICs) into the scaffold
- Test optimally seeded valves in the bioreactor
- Implantation of seeded valves in large animals (pig, sheep) in pulmonary and aortic positions

APPENDICES

6.1 Appendix A

Scaffold Preparation Materials and Protocols

6.1.1 Porcine Heart Valve Decellularization Protocol

Materials:

- Orbital shaker (speed:3-4)
- Heart valves (max of 10 per 500mL of solutions)
- ddH_2O
- 70% EtOH
- 10mM TRIS (2.42g TRIS in 2L ddH_2O at **pH:7.4-7.5**)
- 0.05 M NaOH (2g NaOH in 1L 10mM TRIS)
- Decellularization solution (in 1 Liter 10mM TRIS)
    - 0.05% SDS (Sodium Dodecyl Sulfate) (0.5g) – wear a breathing mask
    - 0.5% TRITON X-100 (5 mL)
    - 0.5% Deoxycholic Acid, Sodium Salt (5g) – wear a breathing mask
    - 0.2% EDTA (Ethylenediaminetetra-acetic acid) (2g)
- RNase/DNase (in 1 Liter PBS: 10 tablets in ddH_2O)
    - 360 mU/mL DNase (0.171 mg)
    - 360 mU/mL RNase (3.677 mg)
    - 1.015 g $MgCl_2$
- Sterile Pyrex wide mouth bottles
- Sterile 1x PBS
- 0.1% peracetic acid (3.125 mL peracetic acid in 1 L PBS at **pH:7.4**)

Methods:

1. Harvest Heart Valves
   -Collect entire mitral valve and plenty of endocardium to clamp for mounting
   -Rinse in ddH_2O 1x (usually in a sample cup)
   -Transport in ddH_2O (usually in a wide mouthed jar)
2. Clean valves over ice
   -Leave mitral valve and endocardium with a very thin layer of muscle tissue
3. Rinse with ddH_2O
4. Incubate in ddH_2O **overnight** at *4°C* in refrigerator

---

5. Rinse with ddH_2O (3x)
6. Incubate in 70% EtOH **20 minutes** at *room temperature* on a shaker
7. Rinse with ddH_2O (3x)
8. Incubate in 0.05 M NaOH **2 hours** at *room temperature* on a shaker
9. Rinse with ddH_2O (3x)
10. Incubate in decellularization solution **overnight** at *room temperature* on a shaker

---

11. Rinse with ddH_2O (5x)
12. Incubate in ddH_2O **5 minutes** at *room temperature* on a shaker (3x)
13. Rinse with ddH_2O (5x or until no bubbles remaining)
14. Incubate in 70% EtOH **20 minutes** at *room temperature* on a shaker
15. Rinse with ddH_2O (3x)
16. Incubate in ddH_2O **2 hours** at *room temperature* on a shaker
17. Incubate in RNase/DNase **overnight** at 37°*C* (oven, incubator, or water bath) on a shaker

---

18. Rinse with ddH_2O (3x)
19. Incubate in 70% EtOH **overnight** at *room temperature* on a shaker

---

*******STERILE CONDITIONS: BOTTLE, SOLUTIONS, ATMOSPHERE*******

20. Rinse with sterile ddH_2O (1x)
21. Incubate in sterile ddH_2O **15 minutes** at *room temperature* on a shaker (2x)
22. Incubate in sterile PBS **30 minutes** at *room temperature* on a shaker
23. Rinse with sterile PBS (2x)
24. Transfer to new sterile bottle
25. Incubate in a sterile bottle in 0.1% peracetic acid **2 hours** at *room temperature* on a shaker
26. Incubate in sterile PBS **15 minutes** at *room temperature* on a shaker (3x)
27. Transfer to new sterile bottles

6.1.2 Porcine Heart Valve Tissue Fixation Protocol

Materials:

- Sterile ½ cotton balls
- Sterile scalpels, scissors, & multiple forceps (for packing cusps)
- Two sterile forceps (for unpacking cusps)
- Saline (7.2g NaCl in 800 mL ddH_2O)
- 0.075% PGG (Slowly add 200mL PGG solution to 800 mL phosphate buffer then run through 0.22 µM sterile filter)
    - Phosphate Buffer - 50mM Na_2HPO_4 (5.68g Na_2HPO_4 in 800 mL saline, **pH: 5.5**)
    - 0. 750g PGG dissolved in 200 mL isopropanol

Methods:

*******STERILE CONDITIONS: BOTTLE, SOLUTIONS, ATMOSPHERE*******

28. Pack cusps in proper closed position with sterile cotton balls soaked in 0.075% PGG
29. Incubate in 0.075% PGG **overnight** at *room temperature* on a shaker

---

30. Remove cotton balls with sterile tools
31. Rinse with sterile PBS (2x)
32. Transfer to new sterile bottle

33. Incubate in sterile PBS **2 hours** at *room temperature* on a shaker
34. Rinse with sterile PBS (2x)
35. Cut aorta at about 3mm above the sinuses
    -Place part of cut aorta on LB agar and other part in liquid broth
    -Wait 3 days and look for turbidity before starting experiment
    Store in sterile PBS at *4°C* up to 2 weeks (optional)

6.2 Appendix B

Porcine Aortic Endothelial Cell Culture Materials and Protocols

6.2.1 Fibronectin Coating of Cell Culture Flask Protocol

Desired concentration: 1 µg FN/cm^2

FN (Sigma F1141) 1mg/mL (=1 µg/µL)
- dilute to 20 µg FN/mL PBS w/o Ca^{++} or Mg^{++}
- apply 50 µL/cm^2 (1 µg FN/cm^2)
- leave flask or plate overnight in the hood (open)
- add medium and plate the cells

Flask	µL FN	µL PBS	Total Volume (µL)
T-25	25	1225	1250
T-75	75	3675	3750
T-150	150	7350	7500

Note: Lower PBS volumes may be needed for timely evaporation

6.2.2 Cell Culture and Confluent Cell Passaging Protocol

Materials:
- Sterile 1X PBS w/o calcium & magnesium (cat#:21-031-CM)
- Trypsin EDTA, 1X (cat#: 25-053-CI)
- Cell culture medium (M199 + 10% FBS + 1% P/S/A)
- Incubator (37ºC±1ºC, 5% CO_2)
- Water bath (37ºC±1.0ºC)
- Centrifuge with appropriate rotor and buckets and set at 1000 RPM

Methods:
1. Warm the trypsin and culture medium to 37 ºC in a water bath (15^+ minutes)
2. Move culture flask from the incubator to the cell culture hood
   ***NOTE: not advisable to do > 4 at one time

3. Aspirate the culture medium with glass pasture pipette
4. Rinse the culture flask with sterile PBS then aspirate
   5mL for T-25 flask
   15mL for T-75 flask
   30mL for T-150 flask
5. Transfer trypsin into the flask
   2mL for T-25 flask
   6mL for T-75 flask
   12mL for T-150 flask
6. Ensure that the flask bottom is completely covered
7. Leave the flasks stationary for 10-20 seconds
8. Aspirate all but a few drops (~1mL) of the trypsin
9. Monitor the progress at room temperature under the microscope until cells round up
10. Release the rounded cells from the flask surface by hitting the side of the flask against your palm (don't splash on lid) until most of the cells are detached
    ***NOTE: leave cells in trypsin for as little time as possible (trypsin kills cells)
    ***NOTE: If the cells detach without hitting, it means the cells are over trypsinized
11. Add 1-2 times the amount of culture medium into the flask as was added for the trypsin to inactivate the trypsin (the lower end of the range works well and saves medium)
    2.5-5mL for T-25 flask
    7.5-15mL for T-75 flask
    15-30mL for T-150 flask
12. Disperse the cells by gently pipetting up and down a few times
13. Transfer the entire mixture into a conical tube
14. OPTIONAL: Rinse the flask with culture medium and transfer the solution into the same conical tube
    2.5mL for T-25 flask
    7.5mL for T-75 flask
    15mL for T-150 flask
15. Examine the flask under a microscope. If there are >20% cells left, repeat steps 4-14
16. Spin down the cells at 220 x g (1000 RPM) for 5-7 minutes
17. Label a new flask while your cells are spinning with the cell line, passage number, date, and your initials
18. Aspirate culture medium from conical tube – be sure not to disturb the pellet
19. Add appropriate volume of culture medium to the conical tube to get approximately $0.25x10^6$ cells/mL (the Scepter ^{TM} is accurate between $0.05x10^6$ and $0.5x10^6$ cells/mL)
20. Break up the pellet at the bottom of the conical tube by gently pipetting up and down
    ***NOTE: Avoid Bubbles
21. Transfer 150 µL of the cell suspension to a 1.5mL microcentrifuge tube
22. Outside the hood, count the cells using the Scepter ^{TM}

23. Add the volume of culture medium that you will need (minus the volume of resuspended cells) depending on your flask size into each flask
    ~4mL for T-25 (5mL total)
    ~13mL for T-75 (15mL total)
    ~26mL for T-150 (30mL total)
24. Add cell suspension into the flask
    5,000 cells/cm^2 cells for regular subculturing ($0.375x10^6$ cells in a T-75 flask)
    15,000 cells/cm^2 cells for rapid growth ($1.125x10^6$ cells in a T-75 flask)
25. Evenly distribute the cells on the flask by "10 figure-8 movements and 4 rotations"
26. Put the flask into the incubator
27. Aspirate and add fresh medium every 2 days (3 on the weekend with increased medium volume)
    5mL for T-25 flask
    15mL for T-75 flask
    30mL for T-150 flask

Passage the cells at 80% confluence

6.2.3 Cell Counting Protocol

Scepter ™ by Millipore (Millipore 2011)

- Turn on the Scepter™ cytometer by pressing the control button on the back of the instrument and wait for on-screen instructions to appear.
- When prompted, attach a sensor to the end of the Scepter™ unit with the electrode sensing panel facing toward the front of the instrument, and you'll see detailed instructions for each step of the counting process.
- Pipette once to draw sample into the sensor. 50 uL of your cell suspension is drawn into the microfabricated, precision-engineered channel embedded in the sensor. The cell sensing zone detects each cell drawn into the sensor and thus cell concentration is calculated.

6.2.4 Porcine Aortic Endothelial Cell Seeding Protocol

Methods

Prior to seeding day prep

- Culture necessary quantity of Cells for experiment (3hv*Xx10^6cells=Xmillion cells)
- **Sterilize Seeding chambers (12hr), bioreactor (12hr), and mounting tools (1hr)**
- Reserve time for Confocal and SEM
- Prepare 500ml of MCDB 131 medium (trypsinizing and seeding volume)

- Label 15 mL conical tubes [(3cusp+3aortic wall)*3Time Points{TP}=18] (4 Karnovske +2 L/D per HV)
    - -2hr cusp L/D, 2hr cusp Karnovske SEM, 2hr cusp Karnovske Confocal, 2hr Aorta L/D, 2hr Aorta Karnovske SEM, 2hr Aorta Karnovske Confocal
    - -Repeat for 4 and 6 hr TP

****1 Day prior to seeding**

Sterile P-cup in sterile conditions.
Make 5% Albumin (5g/100mL PBS) and filter. Keep in fridge.
Rinse P-cup with 5% Albumin and slow shake for 1 hour
Remove 5% Albumin solution while maintaining sterility
Place HV in P-cup add Fibronectin solution (150ug/80ml)
Place on rotisserie overnight.

Seeding Day Prep

- Make Karnovske solution (5ml/sample*12 15mL conical tubes= 60ml)
- Make L/D solution just prior to each TP (~500uL/sample*2 15mL conical=1ml/TP)
- Take Lots of Pictures throughout process

Seeding Procedure

1. Trypsinize 4 T-150 flasks
2. Combine cells into 50mL conical tube, spin down and re-suspend in 5mL of medium. Count cells using ceptor.
    - -If less than 30x10^6cells (10million*3HV) trypsinize another flask
    - -Calculate amount of cell suspension solution needed for 8x10^6 cells per valve
3. Mount 3 Valves in mounting rings and seeding chambers.
4. Fill each chamber with ~100ml of medium.
5. Add counted/calculated cells to each chamber.
6. Place on shaker frame in incubator at 5%CO2 and 37C
7. Set Program parameters and Run Program
    - Shaker ~3; 15 min CW and CCW rotations; 5 min Pause btw cycles.
    - During paused interval rotate chambers 120° CW (looking through the top)
8. After 6 hrs passes.
9. Remove chambers from frame and move into incubator.
10. Leave in incubator **overnight** with sterile filter attached
11. Make medium for bioreactor
12. Move seeding chambers from incubator to hood.
13. Prepare L/D solution
14. Remove one valve and mount in the bioreactor for mechanical stimulation
    - Place in incubator for 2 week incubation

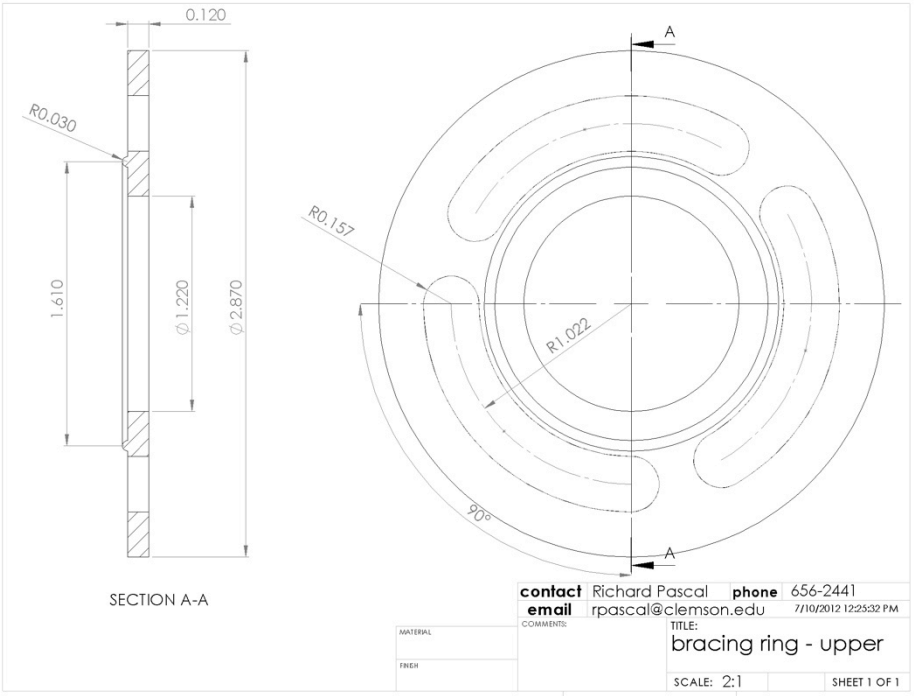
15. Remove second valve and prep for immediate L/D, SEM
16. Cut out cusps and aortic samples and place in labeled conical tubes for analysis.
17. Look at L/D under microscope and take pictures.
18. Count cells remaining in suspension solution.
19. Analyze Karnovske fixed tissue at scheduled SEM and confocal times.

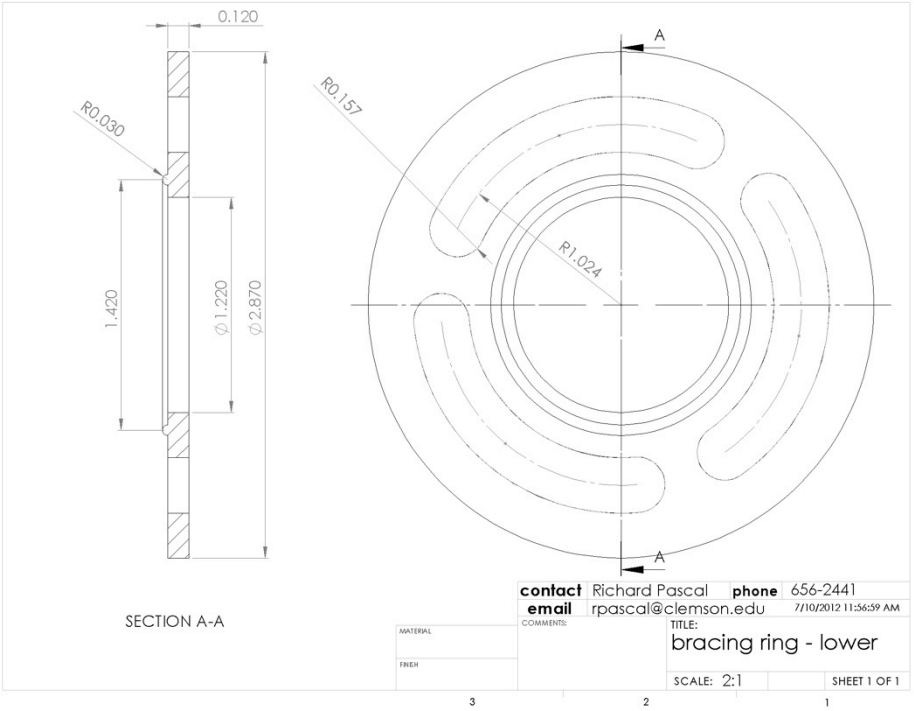
---(1-2week bioreactor conditioning)

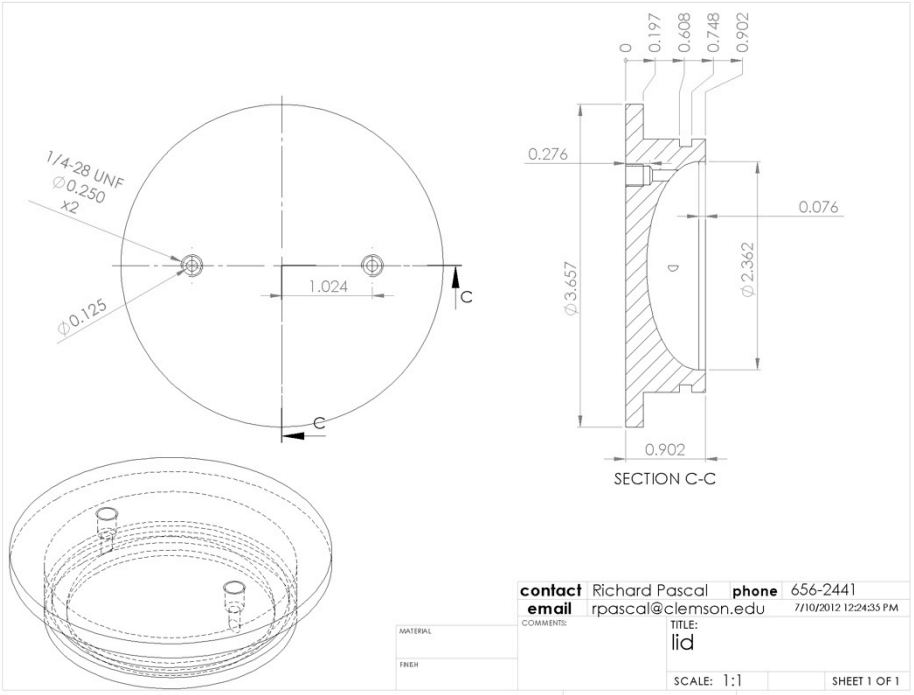
20. Change bioreactor medium every 3 days
21. After 1-2 wk time point analyze valve as described in step 14 above

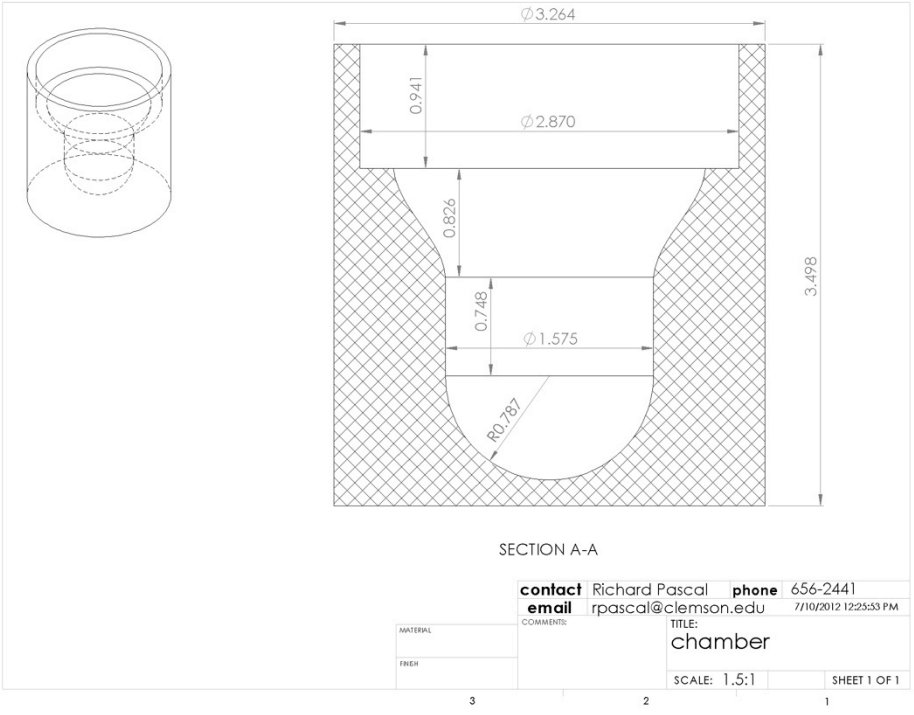
6.3 Appendix C

Dynamic Seeding Device Design Drawings






1/4-28 UNF
⌀0.250
x2
⌀0.125
1.024
C
C
0
0.197
0.608
0.748
0.902
0.276
0.076
⌀3.657
⌀2.362
0.902
SECTION C-C
contact Richard Pascal phone 656-2441
email rpascal@clemson.edu 7/10/2012 12:24:35 PM
COMMENTS:
MATERIAL
FINISH
TITLE:
lid
SCALE: 1:1
SHEET 1 OF 1
3
2
1


⌀3.264
0.941
⌀2.870
0.826
0.748
⌀1.575
R0.787
3.498
SECTION A-A
contact Richard Pascal phone 656-2441
email rpascal@clemson.edu 7/10/2012 12:25:53 PM
COMMENTS:
MATERIAL
FINISH
TITLE:
chamber
SCALE: 1.5:1
SHEET 1 OF 1
3
2
1

6.4 Appendix D

Seeded Scaffold Analysis Materials and Protocols

6.4.1 Live/Dead Assay Protocol

Materials (for 10mL):

- 1x PBS
- Six-well culture plates
- Live/DEAD® Viability/Cytotoxicity Assay Solution (Molecular Probes) (Vortex the EthD-1 and PBS, then add calcein and vortex)
    - 20 µL EthD-1
    - 5 µL 4mM calcein
    - 10mL 1x PBS

Methods:

1. Dissect valve leaflet from the valve
2. Rinse leaflet with PBS (1x)
3. Place leaflet in a well of the six-well culture plate
4. Add ~3mL stain to each well
5. Incubate in Live/DEAD® solution **20 minutes** at *37°C* in the dark
6. Image using FITC and Texas Red filters to examine the cells

6.4.2 Scanning Electron Microscopy (SEM) Sample Preparation Protocol

SEM Sample Preparation
Materials:

- Karnovsky's Fixative (Make and pH caco buffer before adding GA and FA)
    - 0.1M Cacodylic Acid in ddH_2O at pH: 7.4
    - 2.5% Glutaraldehyde (EM Grade)
    - 2.0% Formaldehyde (From 36.5% – NOT phosphate buffered)
- EM Grade Ethanol
- ddH_2O
- Hexamethyldisilazane (HMDS)

Methods:

36. Fix samples in Karnovsky's fixative **overnight** at *room temperature*
    -Use plenty of fixative to ensure complete fixation
37. Dehydrate in Ethanol (adjust time as necessary for thin/thick tissues)
    -50% EtOH 20 minutes
    -70% EtOH 20 minutes
    -85% EtOH 20 minutes

-95% EtOH 20 minutes
-100% EtOH 30 minutes
-100% EtOH 30 minutes
-May hold in 100% EtOH **overnight** at *4°C* if needed

38. Critical point dry
-HMDS 20 minutes
-Aspirate HMDS
-Allow samples to air dry
39. Mount on aluminum stubs with double sided cabon tape
40. Sputter coat 2 minutes with platinum (Hummer 6.2) following instructions in folder.
41. Mount platinum coated samples in Hitachi TM-3000 and image.

REFERENCES

Anatech USA (2012) Hummer® 6.2 Sputter Coater For SEM Sample Preparation.

Apte S, Paul A, Prakash S (2011) Current developments in the tissue engineering of autologous heart valves: moving towards clinical use. Future Cardiology 7:77-97. DOI: 10.2217/fca.10.120

Arrigoni C, Chittò A, Mantero S, Remuzzi A (2008) Rotating versus perfusion bioreactor for the culture of engineered vascular constructs based on hyaluronic acid. Biotechnology and Bioengineering 100:988-97. DOI: 10.1002/bit.21828

Burg K, Holder W, Culberson C, Beiler R, Greene K, Loebsack A, Roland W, Eiselt P, Mooney D, Halberstadt C (2000) Comparative study of seeding methods for three-dimensional polymeric scaffolds. Journal of Biomedical Materials Research 52:576

Butcher JT, Nerem RM (2007) Valvular endothelial cells and the mechanoregulation of valvular pathology. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 362:1445-57. DOI: 10.1098/rstb.2007.2127

Butcher JT, Nerem RM (2006) Valvular endothelial cells regulate the phenotype of interstitial cells in co-culture: effects of steady shear stress. Tissue Engineering 12:905-15. DOI: 10.1089/ten.2006.12.905

Cell Applications Inc. (2005) Porcine Aortic Endothelial Cells (PAOEC).

Chester AH, Taylor PM (2007) Molecular and functional characteristics of heart-valve interstitial cells. Philosophical transactions of the Royal Society of London. Series B, Biological Sciences 362:1437-43. DOI: 10.1098/rstb.2007.2126

Chuang T-H, Stabler C, Simionescu A, Simionescu DT (2009) Polyphenol-stabilized tubular elastin scaffolds for tissue engineered vascular grafts. Tissue Engineering. Part A 15:2837-51. DOI: 10.1089/ten.TEA.2008.0394

Dong N, Shi J, Hu P, Chen S, Hong H (2008) Current progress on scaffolds of tissue engineering heart valves. Frontiers of Medicine in China 2:229-234. DOI: 10.1007/s11684-008-0043-9

Gandaglia A, Bagno A, Naso F, Spina M, Gerosa G (2010) Cells, scaffolds and bioreactors for tissue-engineered heart valves: a journey from basic concepts to contemporary developmental innovations. European Journal of Cardio-thoracic Surgery 39:523-531. DOI: 10.1016/j.ejcts.2010.07.030

Griffon DJ, Abulencia JP, Ragetly GR, Fredericks LP, Chaieb S (2011) A comparative study of seeding techniques and three-dimensional matrices for mesenchymal cell attachment. Journal of Tissue Engineering and Regenerative Medicine 5:169-79. DOI: 10.1002/term.302

Gulbins H, Pritisanac A, Uhlig A, Goldemund A, Meiser BM, Reichart B, Daebritz S (2005) Seeding of human endothelial cells on valve containing aortic mini-roots: development of a seeding device and procedure. The Annals of Thoracic Surgery 79:2119-26. DOI: 10.1016/j.athoracsur.2004.05.085

Heart Lung and Blood Institute N (2011) What Is Heart Valve Disease?

Hecker L, Birla RK (2007) Engineering the heart piece by piece: state of the art in cardiac tissue engineering. Regenerative Medicine 2:125-44. DOI: 10.2217/17460751.2.2.125

Hitachi High-technologies (2010) Instruction manual for model TM3000 tabletop microscope.

Huang S-D, Liu X-H, Bai C-G, Lu F-L, Yuan Y, Gong D-J, Xu Z-Y (2007) Synergistic effect of fibronectin and hepatocyte growth factor on stable cell-matrix adhesion, re-endothelialization, and reconstitution in developing tissue-engineered heart valves. Heart and Vessels 22:116-22. DOI: 10.1007/s00380-006-0940-2

Invitrogen (2006). DAPI Nucleic Acid Stain. M. Probes. Eugene, OR, Molecular Probes**:** 5.

Isenburg JC, Simionescu DT, Vyavahare NR (2004) Elastin stabilization in cardiovascular implants: improved resistance to enzymatic degradation by treatment with tannic acid. Biomaterials 25:3293-302. DOI: 10.1016/j.biomaterials.2003.10.001

Jaron D, Lelkes P, Seliktar R, Hon S-MC, Gerbino R, Gostola N (2008) Types of Heart Valves.

Lichtenberg A, Tudorache I, Cebotari S, Ringes-Lichtenberg S, Sturz G, Hoeffler K, Hurscheler C, Brandes G, Hilfiker A, Haverich A (2006) In vitro re-endothelialization of detergent decellularized heart valves under simulated physiological dynamic conditions. Biomaterials 27:4221-9. DOI: 10.1016/j.biomaterials.2006.03.047

Life Technologies (2005) LIVE/DEAD ® Viability/Cytotoxicity Kit *for mammalian cells*.

Lueders C, Sodian R, Shakibaei M, Hetzer R (2006) Short-term culture of human neonatal myofibroblasts seeded using a novel three-dimensional rotary seeding device. ASAIO Journal 52:310-4. DOI: 10.1097/01.mat.0000217792.45523.bb

Mendelson K, Schoen FJ (2006) Heart valve tissue engineering: concepts, approaches, progress, and challenges. Annals of Biomedical Engineering 34:1799-819. DOI: 10.1007/s10439-006-9163-z

Millipore (2011) Scepter ^{TM} Handheld Automated Cell Counter User Guide.

Mirensky TL, Hibino N, Sawh-Martinez RF, Yi T, Villalona G, Shinoka T, Breuer CK (2010) Tissue-engineered vascular grafts: does cell seeding matter? Journal of Pediatric Surgery 45:1299-305. DOI: 10.1016/j.jpedsurg.2010.02.102

Nieponice A, Soletti L, Guan J, Deasy BM, Huard J, Wagner WR, Vorp D a (2008) Development of a tissue-engineered vascular graft combining a biodegradable scaffold, muscle-derived stem cells and a rotational vacuum seeding technique. Biomaterials 29:825-33. DOI: 10.1016/j.biomaterials.2007.10.044

Ouyang A, Yang S-tian (2007) Effects of mixing intensity on cell seeding and proliferation in three-dimensional fibrous matrices. Biotechnology and Bioengineering 96:371-80. DOI: 10.1002/bit.21091

Scheve T (2008) What's aortic valve disease?

Shimizu K, Ito A, Arinobe M, Murase Y, Iwata Y, Narita Y, Kagami H, Ueda M, Honda H (2007) Effective cell-seeding technique using magnetite nanoparticles and magnetic force onto decellularized blood vessels for vascular tissue engineering. Journal of Bioscience and Bioengineering 103:472-8. DOI: 10.1263/jbb.103.472

Sierad LN, Simionescu A, Albers C, Chen J, Maivelett J, Tedder Mary Elizabeth, Liao J, Simionescu DT (2010) Design and Testing of a Pulsatile Conditioning System for Dynamic Endothelialization of Polyphenol-Stabilized Tissue Engineered Heart Valves. Cardiovascular Engineering and Technology 1:138-153. DOI: 10.1007/s13239-010-0014-6

Sung HW, Huang RN, Huang LL, Tsai CC, Chiu CT (1998) Feasibility study of a natural crosslinking reagent for biological tissue fixation. Journal of Biomedical Materials Research 42:560-7

Tedder Mary E, Liao J, Weed B, Stabler C, Zhang H, Simionescu A, Simionescu DT (2009) Stabilized collagen scaffolds for heart valve tissue engineering. Tissue Engineering. Part A 15:1257-68. DOI: 10.1089/ten.tea.2008.0263

Tillquist MN, Maddox TM (2011) Cardiac crossroads: deciding between mechanical or bioprosthetic heart valve replacement. Patient Preference and Adherence 5:91-9. DOI: 10.2147/PPA.S16420

Villalona G a, Udelsman B, Duncan DR, et al. (2010) Cell-seeding techniques in vascular tissue engineering. Tissue Engineering. Part B, Reviews 16:341-50. DOI: 10.1089/ten.TEB.2009.0527

Weinand C, Xu JW, Peretti GM, Bonassar LJ, Gill TJ (2009) Conditions affecting cell seeding onto three-dimensional scaffolds for cellular-based biodegradable implants. Journal of Biomedical Materials Research. Part B, Applied Biomaterials 91:80-7. DOI: 10.1002/jbm.b.31376